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Local Activation of Dendritic Cells Alters the Pathogenesis of Autoimmune Disease in the Retina

Neal D. Heuss,*1 Ute Lehmann,*1 Christopher C. Norbury,† Scott W. McPherson,* and Dale S. Gregerson*

Interest in the identities, properties, functions, and origins of local APC in CNS tissues is growing. We recently reported that dendritic cells (DC) distinct from microglia were present in quiescent retina and rapidly responded to injured neurons. In this study, the disease-promoting and regulatory contributions of these APC in experimental autoimmune uveoretinitis (EAU) were examined. APC activity of DC from quiescent retina showed that they promoted generation of Foxp3+ T cells and inhibited activation of naive T cells by splenic DC and Ag. Conversely, in vitro assays of DC purifed from injured retina demonstrated an enhanced ability to activate T cells and reduced induction of Foxp3+ T cells. These findings were supported by the observation that in situ activation of DC before adoptive transfer of β-galactosidase–specific T cells dramatically increased severity and incidence of EAU. Recruitment of T cells into retina by local delivery of Ag in vivo showed that quiescent retina promoted development of parenchymal Foxp3+ T cells, but assays of preinjured retina did not. Together, these results demonstrated that local conditions in the retina determined APC function and affected the pathogenesis of EAU by both CD4 and CD8 T cells. The Journal of Immunology, 2012, 188: 000–000.

A central event in adaptive immunity is T cell activation by presentation of cognate Ag. The APC that perform this function, whether for activation of naive T cells in lymphoid tissues or for presentation of Ag to effector T cells in peripheral tissues, are increasingly found to be dendritic cells (DC) (1, 2). Several animal models are used to study immune responses in nervous system tissues. Most are based on autoreactive CD4 or CD8 T cells specific for neural Ags and include experimental autoimmune uveoretinitis (EAU) (3–6) and experimental autoimmune encephalomyelitis (7). The APC present in the initial stages of immune recognition in the CNS, especially retina, are not yet defined, but candidates have been identified (8–12).

The ability of the intraocular environment to modulate immune responses was an early, paradigm-setting example of tissue-/organ-specific effects on the immune system (13). An important mechanism of ocular immune privilege, that is, immune deviation, is local recognition of Ag and ongoing T cell activation, but the tissue-dependent control of immune effector responses has been proposed to be widespread, allowing immune responses throughout the body to be adapted to the needs of the tissue (15).

Recently, we found that CD45+ cells isolated from quiescent retina, largely microglia (MG), had little ability to present Ag to naive T cells in vitro as measured by T cell activation, proliferation, and cytokine production (16). Presentation of Ag to Ag-experienced T cells was greater than to naive T cells but was still limited. The induction of EAU in the retina implies that there is local recognition of Ag and ongoing T cell activation, but the nature, identity, and origin of the APC are uncertain. Previously, we showed that recruited APC could support the induction of EAU (17). The finding that recruited APC could support the induction of autoimmune T cells suggests that resident retinal APC, including resident DC, are either less able to trigger a damaging T cell response, or that they possess a regulatory phenotype that reduces the potency of autoimmune T cell activity.

In this study, we examine the ability of peripheral DC, resident MG, or retinal DC to activate effector T cell populations and induce EAU, and to stimulate differentiation of Foxp3+ regulatory T cells (Tregs) that can modulate EAU development. We demonstrated that peripheral DC injected into the retina increased the induction of EAU mediated by adoptive transfer of activated T cells, but that resident DC in quiescent retina suppressed the onset of EAU, likely via the induction of Tregs that modulated the activity of T cells specific for retinal Ag. Recruitment of endogenous, activated DC into the retina by local injury promoted the pathogenesis of EAU.

Materials and Methods

**Mice**

Arrβgal mice on the B6 and B10.A backgrounds express β-galactosidase (βgal) under control of the rod photoreceptor arrestin promoter, resulting in βgal expression in retina (150–200 ng), pineal gland (<0.5 ng), and rare, unidentified brain cells (18–20). CD11c-diphtheria toxin receptor (DTR)/GFP breeders were a gift from Dr. S.J. McSorley (University of
These mice express GFP and the DTR using the CD11c promoter on the B6 background (12, 21). CD11c-DTR/GFP mice were also crossed to the B10.A background and F₁ offspring used. Two strains producing βgal-specific TCR transgenic (Tg) T cells were used. βgalTCR mice (B10.A) produce DC T cells specific for βgal (20, 22). BG2 mice express a βgal-specific TCR on B6 DC T cells (23). Breeder pairs of Tg mice expressing GFP driven by the Foxp3 promoter (Foxp3-GFP mice) (24) were a gift from Dr. S. S. Way. Mice were 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 Institutional Animal Care and Use Committee guidelines.

Optic nerve crush injury
A unilateral optic nerve crush (ONC) was performed as previously described (12, 25).

Intraocular injections
Anterior chamber (AC) inoculations were done by transcorneal deposition into the AC of the eye (12). A 33-gauge blunt cannula on a 10-μl Hamilton syringe was used to slowly pass 1.0–1.5 μl saline, diphtheria toxin (5 ng), Ag (5 μg), or cells into the AC.

Generation, activation, and transfer of polyclonal βgal-specific CD8 T cells
The β4 CD8 T cell line specific for an H-2Ld-restricted epitope of βgal (TPHPARIGL) (26) was prepared from female B10.A mice after infection with recombinant, βgal-expressing vaccinia virus (4, 5). For adoptive transfer, recipient mice received 1–15 × 10⁶ activated T cells, as indicated, purified by positive selection for CD90+ cells by magnetic sorting (MACS, Miltenyi Biotech). For AC injections, the T cells were delivered in 1 μl saline.

Preparation of CD4 T cells for adoptive transfer
TCR Tg T cells from pooled spleen and lymph nodes were stimulated with recombinant, βgal-expressing vaccinia virus (4, 5). For adoptive transfer, recipient mice received 1–15 × 10⁶ activated T cells, as indicated, purified by positive selection for CD90+ cells using MACS.

Preparation of splenic or bone marrow APC for AC injection
Fresh CD11c+ DC were prepared from bone marrow (BM) by positive selection with CD11c MACS and an LS column. CD11b+ monocytes were enriched from a suspension of BM cells that were first depleted with mAb to CD19 and CD11c using MACS and LD columns. The flow-through cells were positively selected using anti-CD11b MACS and passage over LS columns. Candidate APC were not activated before AC injection.

Preparation of retinal APC
Retinal DC and MG from naïve CD11c-DTR/GFP mice or from mice pretreated with a retinal injury to recruit and activate myeloid cells in retina. Mice were euthanized and perfused with 12 ml saline. The retinas were removed as described previously (27). Retinas were dissected using 0.5 mg/ml Liberase/Blendzyme3 (Roche) and 0.01% DNAse, washed with Dulbecco’s PBS, incubated for 10 min at room temperature in 0.5 μg anti-mouse CD16/CD32 (Fc block), washed, and labeled with paramagnetic anti-CD45. Following positive selection for CD45 on LS columns, the cells were stained for CD11b and sorted on a FACS Aria (BD Biosciences) for GFP+ cells (DC) and GFP− cells (MG).

In vitro Ag presentation assays
In vitro assays of APC activity were done in 96-well V-bottom plates. βgalTCR CD4 T cells were isolated using a CD4 enrichment MACS kit. Fresh CD11c+ DC were prepared from BM by positive selection with CD11c MACS and an LS column. BG2 CD4 T cells were prepared from BG2 × Foxp3/GFP mice as described earlier and flow sorted for CD4+25 GFP− cells to efficiently remove Tregs. For assays of retinal APC, DC and MG were prepared from CD11c-DTR/GFP mice as described earlier. The purified APC (0–250 cells/well, as indicated) and T cells (5 × 10⁵/well) were combined with or without βgal Ag for 4–5 d, harvested, and stained for CD25, CD44, and CD3. GFP expression (Foxp3 reporter) was detected by its autofluorescence. Activated T cells were defined as CD3+CD44hi CD25+, Tregs were defined as CD3−CD44hi CD25−, βgalTCR CD4 T cells.

Immunostaining of retinal whole mounts
Retinas were removed from euthanized mice (27), fixed in 4% paraformaldehyde for 12 min, and blocked in 10% normal donkey serum (Jackson

![Image](http://www.jimmunol.org/)
Immunoresearch) with 0.1% Triton X-100 for 1 h at room temperature. Tissues were incubated for 3 h at 4°C in Alexa 594-conjugated isolectin B4 (Invitrogen) to stain vascular endothelial cells. GFP was visualized by its autofluorescence. Stained retinal flatmounts were examined using conventional epifluorescence microscopy (Leica DM4000B; Leica, Wetzlar, Germany).

Histopathology

The severity of autoimmune damage to the retina was scored using H&E-stained sections from formalin-fixed eyes as described elsewhere (4, 20).

Results

**CD4 T cell-mediated EAU after transfer of APC into the AC of the eye**

Induction of EAU is dependent on the dose of activated autoreactive T cells given by systemic adoptive transfer (3, 28). If Ag presentation in retina by local APC is a limiting factor in EAU immunopathogenesis, then manipulations that increase the number of local DC may promote EAU induction. Fresh, uncultured CD11c+ DC were isolated from BM and injected into the AC of one eye. Fresh CD11c+ cells were used to include functionally uncommitted DC, and were a mixture of ∼75% CD11c+11b- cells and 22% CD11c+11b+ cells (Fig. 1A). The DC were largely MHC class II+ (64%), but expression of CD40, CD80, and F4/80 was associated with the smaller CD11c+11b+ subset (Fig. 1B). The APC activity of purified DC preparations was confirmed in vitro by assays with purified, naive βgal-specific T cells from βgalTCR Tg mice. Purified DC were highly efficient activators of naive T cells, showing activity at only 31 DC/well (Fig. 1C). CD69, CD62L, and CD44 were also examined, but CD25 staining gave the largest difference and remained elevated for the duration of the assay. The yield of activated T cells in these cultures correlated well with the number of DC added to the cultures, demonstrating their APC function after purification.

Graded numbers of fresh DC purified from BM, or saline only, were inoculated in 1 μl into the AC of the right eye (RE) of arrβgal mice, followed by activated, βgal-specific CD4 T cells injected i.v. Because the T cells were given i.v., they circulated through both eyes, whereas only one eye received DC, providing an internal control for each mouse. Mice developed EAU with an average score of 1.3 in control, unmanipulated left eyes (LE; Fig. 1D). Increasingly severe disease was found with the combination of T cells given i.v. with increasing numbers of DC injected into the RE. Fig. 1 shows representative examples of histopathology without (Fig. 1E) and with (Fig. 1F) DC injection. Injection of 10^5 purified DC alone failed to induce EAU (data not shown). Control experiments using CFSE-labeled DC showed that a small fraction of DC reached the retinal parenchyma after AC injection (Supplemental Fig. 1, Supplemental Tables I, II).

Because we previously proposed that circulating monocytes were precursors of recruited APC in EAU (17), fresh CD11b+11c- cells were prepared from BM by positive selection for CD11b after depletion of CD11c+ and CD19+ cells (Fig. 2A). The small number of neutrophils in this preparation have a short half-life in vivo and in vitro, and do not present Ag (29, 30). The cells were 99% CD11b+ and 1% CD11c+. Unlike purified CD11c+ cells, CD11b+11c- cells showed little expression of MHC class II, CD40, F4/80, and CD80 (Fig. 2B). Monocytes prepared in this manner expressed CD11c after 1 wk in vitro with GM-CSF (Fig. 2C). The EAU-inducing activity of the purified monocytes was compared with fresh CD11c+ DC from BM using the AC injection route into the RE of arrβgal mice. They also received an i.v. in-

![FIGURE 2. Purification, phenotype, and EAU-promoting activities of purified CD11b+ BM cells used in AC injections. A, CD11b+ cells from BM were ∼99% pure and substantially free of CD11c+ cells. B, A small percentage of cells expressed a low level of class II, CD80, and F4/80. C, Culture of CD11b+ cells in GM-CSF led to expression of CD11c+ by a majority of the population. D, EAU induction was elevated to a similar degree by AC injection of 7.5 × 10^4 CD11b+CD11c- or CD11c+ cells into the RE followed by i.v. injection of 2 × 10^6 activated βgalTCR T cells into arrβgal mice. Eyes were harvested for histopathology after 13 d (n = 4). E–H, Representative samples of retinal histopathology are shown. Uninjected LE (E) of arrβgal recipient given 7.5 × 10^4 CD11c+ cells in the RE (F). Uninjected LE (G) of arrβgal recipient given 7.5 × 10^4 CD11b+ cells in the RE (H). H&E stained; original magnification ×20.](http://www.jimmunol.org/)
jection of in vitro-activated, βgal-specific CD4 T cells. Eyes receiving the monocyte-enriched preparation developed EAU similar to those receiving DC (Fig. 2D). Eyes that did not receive either APC preparation developed minimal EAU (Fig. 2D). Equivalent levels of EAU were found after i.v. injection of T cells and AC injection of either CD11c⁺ or CD11b⁺ APC (Fig. 2F, 2H). These results suggested that maturation of monocytes into APC could proceed in the posterior segment environment of the retina. Injection of DC only did not evoke EAU and showed that EAU was T cell dependent. Coinjection of Con A-activated polyclonal B10.A T cells and DC into the same eye of arrβgal mice gave no EAU, demonstrating that the T cells must be Ag specific to induce EAU (data not shown).

EAU induced by CD8 T cells was enhanced by transfer of APC into the AC

Our previous studies of CD8 T cell-mediated EAU showed that severe photoreceptor cell loss was found, but it was accompanied by much less inflammation than that induced by CD4 T cells (4). Because the target Ag (βgal) was expressed in photoreceptor cells, which express little, if any, MHC class I, a potential explanation was that Ag presentation for direct cytotoxicity was limiting. The consequences of inoculating exogenous DC directly into the AC was tested. The CD8 T cells were given i.v., but only one eye received DC. βgal⁺ eyes given exogenous DC developed significantly more disease than control eyes (Fig. 3). Transient development of vacuoles in the retinal pigment epithelium was frequently found in cases of severe EAU resulting from CD8 T cells (Fig. 3C). As a control for Ag dependency of the pathogenesis, βgal⁻ B10.A mice were given larger numbers of T cells and DC, but they did not experience development of retinal disease (Fig. 3A).

The Ag dependency of pathogenesis was tested in experiments using DC pulsed with βgal. Ag-pulsed DC did not induce a higher level of EAU than DC that were not Ag pulsed (Fig. 4A). AC injection of Ag-pulsed DC in combination with βgal-specific CD8 T cells given i.v. into mice lacking retinal βgal gave no EAU. Injection of T cells, DC, and Ag into the same AC of B10.A controls yielded a transient accumulation of immune cells in the angle of the AC, but no retinitis or EAU was observed (data not shown). Clearly, DC promotion of disease was dependent on the presence of target Ag in the retina, the presence of retinal Ag was sufficient to support severe EAU, and addition of exogenous Ag did not support EAU induction in recipients that lack retinal βgal. To determine whether AC injection of T cells and DC would give more severe EAU, we inoculated activated CD8 T cells directly into the AC of one eye of arrβgal mice, with or without DC into the same eye. Eyes receiving only CD8 T cells had an average EAU score of 1.2, whereas eyes receiving both T cells and DC had an average score of 4.3 (Fig. 4B). Adding 5-fold more CD8 T cells did not increase pathology. Because the DC were not loaded with βgal before inoculation, Ag was gathered locally. The doses of T cells and DC were similar to those used when the T cells were given i.v. Similar controls were done using the CD4 βgalTCR T cells and purified DC, with and without Ag. These experiments showed that no EAU was found in the absence of βgal expression in the retina (Fig. 4F).

AC injection of T cells and DC was used to test the dependence of EAU on the dose of APC. Increasing numbers of DC gave more severe CD8 T cell-mediated EAU (Fig. 4C). Even though the mechanisms underlying CD4 versus CD8 T cell-mediated EAU are different, similar numbers of exogenous DC promoted EAU severity for both. Sham injections with saline did not significantly increase histopathology compared with unmanipulated eyes in mice inoculated i.v. with βgal-specific CD4 or CD8 T cells (data not shown). Inoculating CD8 T cells into one eye gave no EAU in the contralateral eye, even though i.v. administration of similar numbers of T cells usually leads to EAU in both eyes. Placing T cells in one eye and the DC in the contralateral eye also gave no EAU in the contralateral eye (data not shown). The data suggested that the intraocular environment had a substantial impact on the activity of the T cells.

Detection and identification of retinal cells with a DC phenotype

The earlier results supported our hypothesis that local APC function before EAU onset was limiting or immunoregulatory. Candidates for retinal APC include MG (31, 32) and DC (12). Quiescent mouse retina contains ∼4500 MG/retina that comprise the majority of CD45⁺ cells in retina (12, 33, 34). Small numbers of putative DC, based on staining for MHC class II or 33D1, were seen in retina by immunofluorescence (8, 9, 35), and expression of putative DC, based on staining for MHC class II or 33D1, were seen in retina by immunofluorescence (8, 9, 35), and expression of low levels of CD11c by flow cytometry (16, 33). Because CD11c has been difficult to use as a marker in fixed tissue for immunofluorescence, we used CD11c-DTR/GFP Tg mice, which express GFP and the DTR in a chimeric protein, using a CD11c promoter. In peripheral immune tissues, the GFP⁺ cells of CD11c-DTR/GFP mice were shown to be DC with APC functions (21, 36, 37). The GFP fluorescence can be detected in retina by microscopy, and most of these cells were highly ramified (Fig. 5A). We previously reported that normal retinas contain an average of 95 GFP⁺ DC/retina (12). Live GFP⁺ DC can be flow sorted, based on GFP expression, to a high degree of purity for in vitro assays (Fig. 5B).
T cell activation by retinal APC populations

The APC activity of retinal GFP+ DC was examined in vitro using naive BG2 Foxp3-GFP T cells and βgal as Ag. T cells were flow sorted to remove GFP+ Tregs before use. GFP+ retinal DC from quiescent retina promoted upregulation of Foxp3 in BG2 T cells in cocultures with Ag, consistent with enhanced production of inducible Tregs (Fig. 6A, left panel). Purified MG (GFP−) isolated from quiescent retina promoted T cell survival, but only a small fraction of these cells was activated, and the majority did not express Foxp3, indicating that normal retinal MG did not significantly impact T cell function (Fig. 6A, right panel). In contrast, GFP+ DC isolated from spleen produced a high percentage of activated T cells and few Tregs upon coculture, consistent with our observation that peripheral DC induce βgal-specific T cell activation during induction of EAU (Fig. 6B). Although the yield of activated T cells from these microcultures is small, the activity of the DC isolated from spleen by the same procedures underscores the functional significance and reliability of the results.

To further examine retinal DC function, we cultured splenic DC, or retinal DC from quiescent retina, with BG2 T cells and Ag, individually or together. Splenic DC showed a graded ability to produce T cells with an activated phenotype (Fig. 7). In contrast, neither retinal DC nor retinal MG activated significant numbers of T cells (Fig. 7). However, coculture of retinal DC (60/well) and splenic DC (15/well) ablated the ability of splenic DC to induce T cell activation. Either the retinal DC regulatory activity counteracted the ability of the splenic DC to activate the T cells, perhaps by inducing anergy in T cells, or the T cells produced by exposure to DC from quiescent retina then suppressed the activation of other T cells by the splenic DC.

DC recruitment by injury promotes EAU

Previously, we found that an ONC reversed the inhibitory activity of the endogenous regulatory response to retinal βgal in the ear-swelling assay for delayed type hypersensitivity (DTH) (38). The ONC injures a small fraction of retinal neurons, stimulating an

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**FIGURE 4.** AC inoculation of APC and/or βgal-specific CD8 T cells into the eyes of mice promoted EAU severity. A, Retinal disease was dependent on the presence of βgal in the retina. Preincubation of DC with βgal (0.1 μM peptide TPHPARIGL, 1 h, 37˚C, washed twice) did not increase disease severity and did not lead to immunopathology in βgal− retina. Eyes were harvested at 15 d (n = 4/group). B, Activated CD8 T cells (×10⁶) and/or DC (×10⁴) purified from BM were inoculated together into the AC of the RE (n = 4/group). C, The contribution of the injected DC was dose dependent. Activated CD8 T cells (×10⁶) and increasing numbers of DCs (×10⁴), as indicated, were inoculated into the AC of the RE. D, Pathogenicity was minimal in the absence of exogenous DC. E, Pathogenicity of the T cells was greatly increased by concurrent injection of 6–10 × 10⁴ DC. Eyes were harvested at 15 d for histopathology. H&E stained; original magnification ×20. F, EAU pathogenesis by βgalTCR CD4 T cells and DC after injection into the AC also required expression of the target Ag in the retina to generate retinal inflammation.

**FIGURE 5.** Morphology of GFP+ cells in the retina of CD11c− DTR/GFP mice. A, The ramified appearance dominated and was found in the retinal ganglion cell/nerve fiber layer. Blood vessels were labeled with Alexa 594-conjugated isoelectin B4, DC were detected by their endogenous GFP (original magnification ×40). B, Presort and postsort analyses of the isolation of splenic GFP+ DC for in vitro APC assays.
increase in the number of retinal GFP+ DC and their activation, as detected by upregulation of MHC class II expression. Accordingly, we predicted that the retinal DC injury response to an ONC might enhance EAU induction. An ONC before adoptive transfer of βgal-specific CD8 T cells promoted the pathogenesis of EAU (Fig. 8), consistent with an injury-induced shift in activity of retinal DC from inducing Tregs to supporting pathogenic T cells.

To address whether GFP+ DC or MG from injured retina induced fewer Tregs and more effector T cells, we cocultured naive BG2 Foxp3-GFP T cells with retinal DC or MG purified from injured retina and βgal. In marked contrast with their ability to induce Tregs when purified from quiescent retina, retinal DC isolated from injured retina promoted T cell activation and few upregulated Foxp3 (Fig. 6C, left panel). In contrast, MG from injured retina were more effective producers of Foxp3+ T cells than DC from injured retinas (Fig. 6C, right panel), indicating that these cells gained a regulatory role, whereas DC gained an activating role. We previously reported that GFP+ DC in CD11c-DTR/GFP mice showed moderate recruitment to the contralateral eye after a unilateral ONC (12). When assayed for APC function in vitro, the contralateral GFP+ DC expressed APC activity similar to DC from injured retina (Fig. 6D, left panel). MG isolated from contralateral retinas were most similar in activity to MG from normal retina (Fig. 6D, right panel).

These results suggested experiments to further test the local APC function of retinal DC. An ONC dramatically increased the number of MHC class II+ GFP+ DC in CD11c-DTR/GFP mice, and these cells had APC function in vitro (Fig. 6C). Conversely, GFP+ DC from quiescent retina promoted Treg development in vitro. Preliminary studies showed that quiescent retina, from wild-type (wt) B6 and BG2 mice, contained a small population of parenchymal T cells in the CD45hi population (Fig. 9A1; Table I). The CD3+ cells were αβ T cells; there was little or no evidence for γδ T cells (data not shown) or NK-T cells (Fig. 9A3). NK cells were found in the CD45hi population (Fig. 9A3; Table I). Analysis of Rag-deficient mice revealed the absence of CD3+ cells (Fig. 9A2; Table I), but NK (DX5+) cells were present (Fig. 9A4). Although the number of CD3 T cells was small, control experiments using Rag-deficient mice demonstrated the reliability of their detection by flow cytometry (see Supplemental Fig. 2 for additional details). These results suggested assessment of retinal APC function by examining the T cell response to Ag in retina in vivo.

In comparisons of wt B6 and BG2 TCR Tg mice, injection of 5 μg βgal into the AC of one eye of BG2 TCR Tg mice generated greater numbers of T cells in retinal parenchyma than were found in wt mice that lack βgal-specific BG2 T cells (Fig. 9B, Table II).
In each case, unmanipulated contralateral retina was similar to normal control retina (Table II, and data not shown). Additional control experiments revealed that injection of saline into wt or BG2 mice recruited few T cells to the retina (Supplemental Fig. 2, Table II). Injection of βgal into BG2 mice on the Foxp3-GFP background showed that retinal T cells recruited by βgal injection into quiescent eyes exhibited a higher proportion of Foxp3+CD4+ Tregs than was found in unmanipulated eyes or after saline injection (Fig. 9B2; Table II). Because in vitro assays (Fig. 6C) showed that DC isolated from retina after an ONC had a reduced ability to generate Tregs, βgal was injected into eyes that had been given an ONC 7 d earlier. Parenchymal T cells from these retinas were elevated in total number per retina, but not in their proportion of Foxp3+CD4+ T cells (Fig. 9C1; Table II). Contralateral retinas from these mice are shown in Fig. 9C2. The potential relationships between the resident, recruited, and injected DC are shown in Fig. 10.

**Discussion**

The presence of immune-privileged sites has been recognized since 1948, when Medawar (13) grafted tissue into the AC of the eye and found prolonged survival of tissue in this site. Immune privilege has since been detected in a number of sites and cells, and includes retina and CNS (39, 40). In studies of EAU, a range of factors that affect susceptibility to EAU have been described and reveal that retinal immune privilege is based on a variety of mechanisms (6). Because adaptive immune responses have APC as a common origin, and MHC class II is a factor in EAU susceptibility (41–43), we sought to explore the role of APC in a model using Tg expression of βgal in retinal photoreceptor cells. We chose to exploit the limited susceptibility of B6 and B10.A mice to retinal autoimmune disease on the premise that their resistance to EAU resulted from potent mechanisms of immune privilege. Preliminary trials to determine whether limited susceptibility to EAU could be overcome by transfer of numbers of activated T cells, up to 10–20 × 10^6 T cells/mouse, revealed little increase in disease severity (D. Gregerson and N. Heuss, unpublished observations). Because we found little evidence for retinal cells with Ag-presenting ability in previous studies (16, 33), we proposed that retinal APC function was a constraint on pathogenesis, and sought strategies to locally manipulate APC. We show that provision of exogenous APC by injection of purified DC into the AC of the eye dramatically increased susceptibility of the retina to EAU induced by adoptive transfer of activated CD4 or CD8 T cells specific for βgal. We conclude that the function of retinal DC was an important determinant of the outcome of retinal immune responses.

In studies testing transfer of fresh, BM-derived DC into the eye, promotion of EAU was limited to the eye receiving exogenous APC, and the effect correlated with the number of DC that were inoculated. Although some APC injected into the AC undoubtedly escaped the eye, the strict correlation between EAU and unilateral injection of APC showed that the APC activity critical for pathogenesis was local. If the unilateral APC effect depended on APC migration to secondary lymphoid tissues and generation of T cells, then T cells generated remotely would have free access to both eyes through the circulation and would be unable to limit pathogenesis to the ipsilateral retina. Similarly, increased disease severity was unrelated to T cell access to the retina. Bypassing the circulation by intraocular injection of a number of T cells similar to that given i.v. gave only minimal EAU in the T cell-injected eye unless DC were also coinjected. Injection of a uveitogenic dose of T cells into one...
eye gave no EAU in the opposite eye, suggesting that the local environment altered the activity or migratory properties of the cells. Together, the ipsilateral dependency of EAU pathogenesis underscored the critical role of local Ag presentation.

Direct assay of the APC activity of myeloid cells from retina revealed effects of the retinal environment on their activity. DC residing in quiescent retina of CD11c-DTR/GFP Tg mice were isolated by their expression of GFP on the CD11c promoter, and tested for their ability to present βgal to naïve BG2 T cells depleted of Foxp3+ T cells. T cells recovered from these cultures were ~50% Foxp3+. In contrast, MG isolated from quiescent retina better supported T cell survival in these assays, but only a small portion was activated, and fewer were Foxp3+. Upregulation of DC numbers and activity by the ONC injury gave a much different result in the APC assays; many more T cells were recovered and activated, but only 5% were Foxp3+. The activity of these DC resembled that of GFP+ splenic DC. MG from injured retina were less active but more able to generate Foxp3+ T cells. Together, these results suggested that EAU induction might be more effective after the ONC, and this result was found.

Based on the in vivo and in vitro assays and manipulations of retinal APC activity, a model for the presence and function of DC in the retina was constructed (Fig. 10). DC in normal quiescent retina are present in small numbers and turn over slowly. Unpublished observations (D. Gregerson, N. Heuss, and S. McPherson) suggest they originate from an unidentified circulating precursor. Candidates include monocytes, or a separate lineage of DC precursors, or progenitors that pass through a local niche (pathway 1). In vitro, these cells favored production of Foxp3+ T cells and appeared to inhibit attempts to induce EAU.

DC numbers increased dramatically after a modest retinal injury, and they upregulated class II expression (12). The speed at which large numbers of these cells appeared (several thousand by 5 d after injury) suggests a relatively direct origination from circulating precursors, possibly monocytes (pathway 2). Preliminary results suggest some may also be derived from existing retinal DC or progenitors (pathway 4). In vitro, they preferentially activated T cells, and few were Foxp3+. Their recruitment to the retina restored DTH to retinal autoantigens, and the retinas become more susceptible to EAU. These findings suggest that the retinal milieu modulated their activity so that retinal DC promoted homeostasis in the absence of significant injury but supported a T cell response after injury.

A small portion of DC or monocytes isolated from spleen or BM and injected into the AC entered the retina, bypassing the circulation and extravasation through the vascular endothelium (pathway 3). This route may have produced a critical difference in their function, as they promoted EAU induction in the absence of an injury to the retina. The physiologic extravasation process of precursors entering the quiet retina may enhance their regulatory function; conversely, DC or monocyte precursors that bypassed the extravasation process promoted EAU induction. This route may be little used in normal retina, but the posterior segment of inflamed
The significance of local recognition of a retinal Ag found in this study is consistent with our previous observation that an ONC changed the balance of effector/regulatory activity to a retinal protein. In that study, the endogenous regulatory response to a retinal Ag inhibited the systemic DTH response to that Ag measured by the ear-swelling assay. After an ONC, the regulatory activity was lost, and full-scale ear swelling was found (22). Together, the data suggest that this balance of T cell activity to an Ag in an immune-privileged site is a dynamic process dependent on the nature of the local recognition of Ag. We propose that the small number of DC present in quiescent retina belies their critical role in maintaining the endogenous regulatory response that protects retina from autoimmunity. Their rapid response to stimuli, in which their numbers are rapidly elevated and their function is modified to support T cell activation, further demonstrate a central role in local immune homeostasis of an immune privileged tissue. These rapid changes in response to manipulations suggest a sentinel role. In other tissues, migration to draining lymph nodes would be the likely outcome. For the retina, which lacks lymphatics, their route out of the retina and their destination is currently unclear.

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Disclosures
The authors have no financial conflicts of interest.

References
involves more than a predisposition to generate a T helper-1-like or a T helper-2-like response. *J. Immunol.* 159: 1004–1011.


Supplemental Data; Figure Legends 1 and 2

Supplement Figure 1. CFSE-labeled, BM-derived DC in retinal wholemounts (A,B) and retinal sections (C-E) one day post-AC injection. ONH - optic nerve head; PERI - far peripheral retina; other abbreviations as shown in Supplement Table II. Purified syngeneic DC were labeled with CFSE and injected into the AC of one eye (10^5 DC in 1.5 μl saline) of 12 mice. Mice were harvested at the time points indicated in Supplement Table I, and on day 7 post-injection. Following perfusion fixation with 4 % paraformaldehyde, the retinas were collected for examination as wholemounts for cell counting, or the eyes were taken for frozen sections to allow assessment of their distribution in the layers of the retina. Slides were examined for CFSE-labeled cells. A peak of approximately 1 % of the injected cells was found in the retina at 7 h and 1 d post-injection. By day 7, labeled cells were rare. Retinal wholemounts were prepared as described in the Materials and Methods section. Frozen sections were prepared as described by Ferrington et al., J. Neurochem. 106:158-169, 2008.

Supplement Figure 2. Flow cytometry of control mice showing the presence of CD3^+ and NK (DX5^+) T cells in mouse retina. A. Quiescent mouse retina contained a small population of parenchymal T cells in the CD45^hi population. Normal B6 retinas were harvested, dissociated and assessed by flow cytometry. CD45^hi cells contained CD3^+ α/β T cells and DX5^+ NK cells. Few γδ T cells or NK-T cells were found (data not shown). Myeloid cells, including the DC and microglia (MG), were found in the CD45^med population (not shown). B. Rag-deficient mouse retinas had no CD3^+ T cells, but NK cells were present in the CD45^hi population. The Rag mice provide a useful control for validating the detection of a small number of T cells in the retina. Although the number of CD3^+ T cells in wt mice is small, their detection is not spurious. They were absent in Rag-deficient mice, while the NK population remains. Note that the retina from Rag-deficient mice is normal in other respects, and contained the usual population of myeloid cells in the CD45^med population. The gating on side scatter vs CD45 provides an effective strategy to separate the small number of immune cells from the large populations of neurons and glia. The mice are extensively perfused prior to collecting the retinas.
Supplement Table I. Detection of CFSE-labeled, BM-derived DC in retina following AC injection

<table>
<thead>
<tr>
<th></th>
<th>7 h</th>
<th>d1</th>
<th>d2</th>
<th>d3</th>
<th>d4</th>
</tr>
</thead>
<tbody>
<tr>
<td># cells/field(^b)</td>
<td>9.5 ± 12.2</td>
<td>7.5 ± 7.2</td>
<td>2.3 ± 2.7</td>
<td>1.6 ± 1.7</td>
<td>0.3 ± 1.0</td>
</tr>
<tr>
<td># cells/retina(^c)</td>
<td>1207</td>
<td>952</td>
<td>292</td>
<td>203</td>
<td>38</td>
</tr>
</tbody>
</table>

\(^a\)See legend to Supplement Fig. 1 for experimental details.
\(^b\)Obtained counting cells in retinal wholemounts using a 20x objective, field size 315 x 421 microns.
\(^c\)Calculation based on 127 fields per retina.
Supplemental Data; Table II

Supplement Table II. Distribution of CFSE-labeled cells in the retina at day 1 post-AC injection\textsuperscript{a}

<table>
<thead>
<tr>
<th>Layers of the retina</th>
<th>% of cells/layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ganglion cell/nerve fiber layer GCL/NFL</td>
<td>19%\textsuperscript{b}</td>
</tr>
<tr>
<td>Inner plexiform layer IPL</td>
<td>25%</td>
</tr>
<tr>
<td>Inner nuclear layer INL</td>
<td>13%</td>
</tr>
<tr>
<td>Outer plexiform layer OPL</td>
<td>16%</td>
</tr>
<tr>
<td>Outer nuclear layer ONL</td>
<td>19%</td>
</tr>
<tr>
<td>Photoreceptor inner segments IS</td>
<td>6%</td>
</tr>
<tr>
<td>Photoreceptor outer segments OS</td>
<td>3%</td>
</tr>
</tbody>
</table>

\textsuperscript{a}See legend to Supplement Fig. 1 for experimental details.  
\textsuperscript{b}Counts.