The Antigen-Presenting Activity of Fresh, Adult Parenchymal Microglia and Perivascular Cells from Retina

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*J Immunol* 2004; 172:6587-6597; doi: 10.4049/jimmunol.172.11.6587

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The Antigen-Presenting Activity of Fresh, Adult Parenchymal Microglia and Perivascular Cells from Retina

Dale S. Gregerson, Thien N. Sam, and Scott W. McPherson

Although several observations show local T cell recognition of retinal Ag, there has been no direct demonstration that the APC were retinal derived, rather than recruited. In this study, CD45+ cells isolated from immunologically quiescent murine retina were tested in vitro for functional evidence of Ag presentation to naive and Ag-experienced CD4 T cells specific for β-galactosidase. Because CD45+ cells from brain have been reported to be efficient APC, they were included for comparison. Measures of activation included changes in CD4, CD25, CD44, CD45RB, CD62L, CD69, caspase-3 activation, CFSE dilution, size, number of cells recovered, and cytokine production. Retinal CD45+ cells gave no evidence of Ag-dependent TCR ligation in naive T cells, unlike splenic APC and CD45+ cells from brain, which supported potent responses. Instead, addition of retinal CD45+ cells to cocultures of naive 3E9 T cells plus splenic APC reduced the yield of activated T cells and cytokine production by limiting T cell activation at early time points. Ag-experienced T cells responded weakly to Ag presented by retinal CD45+ cells. Activating the retinal cells with IFN-γ, anti-CD40, or LPS incrementally increased their APC activity. Addition of neutralizing Abs to TGF-β did not reveal suppressed retinal APC activity. Because retina lacks tissue equivalents of meninges and choroid plexus, rich sources of dendritic cells in brain, cells from retina may better represent the APC activity of fresh, adult CNS parenchymal and perivascular cells. The activity of the retinal CD45+ cells appears to be directed to limiting T cell responses. The Journal of Immunology, 2004, 172: 6587–6597.
we examined fresh CD45+ cells isolated from retina of young adult mice, and assessed their functional APC activity using in vitro assays. Retinal CD45+ (RetCD45+) cells displayed a modest ability to stimulate Ag-experienced T cells, but little or no ability to stimulate naive T cells.

Materials and Methods

Mice

Experiments were done with mice on the B10.A or C57BL/6 (B6) background. The 3E9 mice (B10.A) supplied the CD4 T cells. These mice express a Tg TCR specific for a class II MHC-restricted immunodominant epitope of β-galactosidase (β-gal), and were made as described elsewhere (our manuscript in preparation). The 3E9 TCR uses Vβ10. These cells comprise ~80% of the CD3+ cells in lymph nodes (LN). Use of mice received local Institutional Animal Care and Use Committee approval.

Ags and Abs

A synthetic peptide (YYVDEANIEHTGVMV) containing a class II-restricted immunodominant epitope of β-gal was used. All Abs, clones, conjugates, and sources are listed in Table I.

Collection of tissues for preparation of CD45+ cells

Preparation of CD45+ cells from retina is largely as previously described (21), except that the collagenase has been replaced with 75 μl of PBS containing Liberase Blendzyme-3 (1.5 mg/ml; Roche, Indianapolis, IN), and DNase (80 μg/ml) per retina. Collected retinas were warmed to 37°C and mixed gently and frequently to dissociate the tissue. A total of 18–20 retinas was collected for most studies. Brain was dissected free of dura, but much pia remained attached. No attempt was made to exclude choroid plexus. Samples of brain and spleen were dissociated with the same enzymatic procedure. Cell suspensions were diluted with 10 vol of PBS containing 0.5% BSA, 0.5 mM MgCl2, and 80 μg/ml DNase, and incubation was continued for 5 min with gentle mixing. The suspension was applied directly to a density gradient. Cells at the 1.0875 g/cm2 interface were collected for selection of CD45+ cells.

Positive selection of RetCD45+ and brain CD45+ (BrnCD45+) cells

Enrichment of CD45+ cells was done using the MACS magnetic cell separation system (Miltenyi Biotec, Auburn, CA). Cell suspensions were incubated with anti-CD45 conjugated with paramagnetic beads before application to MS+ or LS+ columns, per manufacturer’s recommendations. Retinal cells were positively selected by one or two passages over columns with anti-CD45, as noted in Results.

T cell purification and preparation

To prepare naive T cells, LN and spleen cells from 3E9 TCR Tg mice were collected, pooled, washed, and applied to anti-mouse IgG- and IgM-coated T75 flasks for 1 h at 37°C. The flasks had previously been coated with 0.5 mg of anti-mouse IgG and IgM overnight at 4°C and washed. Nonadherent cells were collected and negatively selected with a mixture of anti-CD8a, anti-CD11b, anti-CD11c, and anti-CD19 on Miltenyi Biotec LD depletion columns. Nonadherent cells were then positively selected with anti-Thy-1.2. In some experiments, the cells were also positively selected with anti-CD4, as specified in Results. Ag-experienced 3E9 T cells were made from LN and spleen from 3E9 TCR Tg mice cultured with β-gal peptide at 10 μM. After 10 days, viable cells were recovered and cultured with irradiated (3000 R) syngeneic spleen, peptide Ag, and 20 U/ml IL-2. Viable cells were again recovered after 10 days and purified on density gradients, followed by positive selection for Thy-1+ cells. The resting, purified T cells were defined as Ag-experienced T cells (AE-T), and used in assays, where indicated.

Cultures of T cells and RetCD45+ or BrnCD45+ cells

Cultures of purified 3E9 T cells and cells being tested for APC activity were mixed, with and without Ag, in V- or U-bottom microculture plates at cell numbers described in Table I. After 20 min on ice, cells that were incubated with biotin-labeled Abs were washed once and resuspended in streptavidin conjugates. After 15 min, all cells were washed once and resuspended for flow cytometry in a FACS Calibur using CellQuest software (BD Biosciences, San Jose, CA). The entire contents of each well were acquired.

Cytokine assays

IL-2 and IFN-γ were assayed by ELISA from the culture supernatants using kits, according to manufacturer’s recommendations (BD PharMingen, San Jose, CA).

Results

Phenotype of RetCD45-positive cells and purified 3E9 T cells

RetCD45+ cells from quiescent retina were purified, as described (Fig. 1A). The cells fall into two populations with respect to CD45 expression levels: intermediate (int) and high. The CD45int population (left column) contains most of the cells that have properties associated with MG. The CD45high cells (right column) displayed little MHC class II, CD11c, or F4/80, especially when compared with the BrnCD45+ cells (see also Fig. 4). A low level of Fas ligand (FasL) was found on both CD45int and CD45high cells. Native T cells were prepared as described with a purity of ~99% (Fig. 1B). The T cells expressed low levels of activation markers, including CD25, CD69, caspase-3-active form, and were CD62Llow.

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Table I. Antibodies

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* BD Biosciences, Mountain View, CA; Miltenyi Biotec, Auburn, CA; Alexis Biochemicals, San Diego, CA; Sigma-Aldrich, St. Louis, MO; R & D Systems, Minneapolis, MN; Caltag, Burlingame, CA.
from 3-day cultures of naive 3E9 T cells (N-T cells) with fresh, nonirradiated SPL as APC without (left) or with (right) Ag. Addition of Ag clearly shifts the viable T cells (events in outlined region) from a small, resting population to large, activated cells. Their CD69 and CD45RB staining (middle pair of panels) shows the expected increase in CD69 and decrease in CD45RB in the presence of Ag. CFSE dilution in the presence of Ag and SPL was found.

The combined data for all culture conditions harvested at 3 days are shown in Fig. 2B. Culture of 3E9 T cells with RetCD45 cells in the presence or absence of Ag and SPL reveals that the retinal cells were unable to induce changes in the T cells that were found with splenic APC controls, i.e., there was no Ag-dependent increase in the recovery of viable T cells, no increase in forward scatter (FSC) or CD69 expression, and no decrease in levels of CD45RB or CFSE. However, evidence for a regulatory effect is found in experiments in which the retinal cells were added to cultures of naive T cells and spleen cells. The combined results of multiple determinations from several experiments show that the number of viable T cells recovered on day 4 is decreased by 25 to 70% (Fig. 2C), but the T cells show similar indications of activation, including an increase in FSC, CFSE dilution, and a decrease in CD45RB expression (Fig. 2B).

In a similar experiment, cultures were harvested at 4 days for flow cytometry, and supernatants were collected for cytokine assays. RetCD45 cells were also unable to induce significant Ag-dependent increases in CD44 or CD25 levels in N-T cells, and did not stimulate IL-2 production (Fig. 3) or IFN-γ production (data not shown). Production of IL-2 was inhibited by addition of syngeneic RetCD45 cells to cultures of N-T cells and splenic APC, but CD44 and CD25 levels were unaffected. Inclusion of RetCD45 cells from B6 mice also inhibited IL-2 production, and had no effect on CD44 and CD25 expression, suggesting that the inhibitory effects were not dependent on ligation of the TCR by peptide/MHC. The presence of RetCD45 cells appears to make the interaction of T cells and splenic APC less productive, in terms of recoverable daughter cells.

**Phenotype of BrnCD45⁺ cells**

Because retina is a CNS tissue, and brain has been reported to be a source of APC, it was important to confirm that result using our cells and protocols. Perfused brain was collected and CD45⁺ cells were isolated, as described. The BrnCD45⁺ cells appeared to be MG (Fig. 4), but several differences were apparent between CD45⁺ cells from brain and retina. Many more Dec-205⁺ cells were present in the preparation from brain, and 97% of them were MHC class II⁺ (data not shown).

**Ag presentation by BrnCD45⁺ cells to naive T cells**

Unlike cells isolated from retina, BrnCD45⁺ cells had substantial APC activity, similar to control cultures containing splenic APC (Fig. 5). The cells proliferated strongly in the presence of Ag without exogenous cytokines and up-regulated CD25 and CD44. In the previous experiment, an inhibitory effect of RetCD45 cells on the response of the T cells to Ag and SPL was found. When the T cells were stimulated with the combination of B10.A SPL and BrnCD45 cells, no decrease in recovery of T cells was found. Addition of BrnCD45 cells derived from B6 mice did not support a response in the 3E9 T cells, showing that compatible class II MHC was required. Addition of B6 BrnCD45 cells to cultures with B10.A SPL did not inhibit the N-T cell response below the level of that shown by splenic APC only. Clearly, cells with DC-like ability to present Ag were present in these isolates, and it seems likely that they were contaminants from the meninges and/or choroid plexus.
Will LPS preactivation of RetCD45+ cells confer Ag-presenting activity for naive T cells?

Up-regulation of surface molecules relevant to Ag presentation and functional increases in APC activity has been reported when CNS CD45+ cells were activated in vitro (27, 29–33). Similar attempts to activate the Ag-presenting capacity of RetCD45 cells were made. RetCD45 cells were incubated with LPS for 2 days and washed, and purified naive 3E9 T cells were added, with and without Ag. The wells were harvested after 3 days for flow cytometry. Representative results are shown in Fig. 6A, including positive and negative controls. LPS activation of the RetCD45 cells led to a 3-fold increase in recovery of 3E9 T cells. Although there was only a modest increase in recovery of viable Vβ10+ 3E9 T cells, CFSE dilution was pronounced, and FSC was clearly increased, indicating activation of the T cells (Fig. 6B). In a parallel experiment, supernatants were harvested after 2 days for cytokine analysis. Controls containing SPL supported IFN-γ production in the presence of Ag, but RetCD45 cells did not support production of IFN-γ in naive 3E9 T cells, with or without LPS preactivation (Fig. 6C).

RetCD45 cells reduce the number of cells that respond to splenic APC

The experiments above demonstrate a dissociation between recovery of activated Vβ10+ T cells and markers of activation and proliferation found when naive T cells are cultured with RetCD45 cells, SPL, and Ag. As shown in Fig. 2, RetCD45 cells that have not been activated had no significant ability to induce changes associated with activation in N-T cells. When LPS activated, there were CFSE dilution and increased FSC without a commensurate increase in recovery of activated N-T cells (Fig. 6) or IFN-γ production. Because a low level of FasL was found on the RetCD45 cells, one explanation for the low recovery of viable N-T cells, despite evidence of activation, could be the induction of apoptosis by RetCD45 cells. Cultures were sampled at early time points for expression of markers of activation (CD69) and apoptosis. As in previous experiments, the number of activated Vβ10+ cells recovered at 96 h was reduced in cocultures containing SPL and RetCD45 cells by ~50% (Fig. 7A, left side). Caspase-3 activation was not significantly altered at any time by the presence of RetCD45 cells, with or without Ag (Fig. 7), providing no evidence that the reduced responsiveness was due to induction of apoptosis in the T cells. The rate of loss of viable 3E9 T cells was unaffected by the presence or absence of RetCD45 cells (data not shown). However, expanding the scale (right side) revealed a reduction in the number of cells that up-regulated CD69 at 24 and 48 h, showing that fewer cells were activated in wells containing RetCD45 cells and Ag at earlier time points (Fig. 7A, right side). This was especially apparent in T cells that had not yet increased in size (Fig. 7B). Several experiments have been done to determine whether RetCD45 cells reduce T cell recovery by inducing apoptosis. We have also done experiments with anti-CD3ε to further test the APC activity of RetCD45 cells, with and without blocking Ab to FasL. None of these studies have given clear evidence that RetCD45 cells inhibit T cell activation by inducing apoptosis (data not shown).

Ag-experienced T cells can be activated by RetCD45 cells, but BrnCD45 are more potent

The activation requirements of Ag-experienced T cells are known to be less stringent than that of naive T cells by several criteria (34), providing a different assessment of the APC activity of RetCD45+ cells. Resting 3E9 T cells, collected 10 days after their most recent activation in vitro, were cultured with RetCD45+ cells, yielding a small increase in the recovery of Vβ10+ cells (Fig. 8). BrnCD45 cells were much more active as APC than the cells isolated from retina. Although the potency of activation found with RetCD45 cells was much less than found with SPL, these cultures produced consistent and unambiguous evidence of Ag-dependent T cell activation, based on a decrease in CFSE intensity and an increase in the number of Vβ10+ 3E9 T cells recovered (Fig. 9). Despite these signs of activation, there was little change in FSC or CD25 expression and little IFN-γ production. Because
TGF-β is known to inhibit APC activation, including MG activation (35, 36), and is expressed at significant levels in retina, neutralizing Ab to TGF-β1 and TGF-β2 was added to some cultures. Neutralization of TGF-β routinely led to a 2-fold increase in recovery of 3E9 T cells in cultures containing splenic APC, but had no significant effect on the cultures testing the APC activity of RetCD45 cells. BrnCD45 cells cultured with AE-T cells under similar conditions were much more active as APC than the cells isolated from retina (data not shown), approaching the activity of the SPL preparation. Mixtures of BrnCD45 cells with SPL did not show the inhibition found when retinal cells were added to SPL/T cell cultures. Instead, the combination of brain and splenic APC was equal to or greater than that of splenic APC alone.

Activated RetCD45 cells show increased ability to present Ag to AE-T cells

The use of APC activators or AE-T cells in the experiments above resulted in increased responses. These findings may reflect conditions in vivo, based on considerations that only activated T cells gain access to the parenchyma of the retina, where they would find local, quiescent APC, and that activated T cells may have some ability to activate local APC due to T cell production and expression of several molecules, including IFN-γ and CD40 ligand (CD40L). In preliminary experiments, fully activated T cells were added to cultures to determine whether the RetCD45 cells, with or without Ag, affected them differentially than addition to SPL cells. These experiments have been unsuccessful to date, as the activated T cells rapidly committed irreversibly to apoptosis during the purification procedures, possibly due to cytokine deprivation and/or loss of other factors.

As an alternative, resting AE-T cells were used. Because the T cells were not active at the time of their addition to the cultures, substitute activators of the RetCD45 cells were added. Two activation strategies were used. In one, the combination of IFN-γ and anti-CD40 was added at the time of coculture of RetCD45 cells, T cells, and Ag. In the other strategy, the RetCD45 cells were pre-incubated with activators for 4 days, and then washed before addition of the T cells and Ag. In both protocols, the cultures were harvested for flow cytometry 3 days after adding the purified, AE-T cells. Two preparations were used as positive controls for APC activity, SPL and CD11c-enriched cells (DC) isolated from spleen by positive selection using anti-CD11c Ab.

Representative scatter plots are shown in Fig. 10. As shown in the combined data (Fig. 10B), evidence for limited Ag stimulation of the AE-T cells was consistently found when activated or pre-activated RetCD45 cells were present. This is reflected in an increased number of recovered T cells (~900 3E9 T cells recovered, compared with 420 recovered cells for cultures with nonactivated retinal cells, and 160 T cells recovered from cultures with no added APC). The increases are not simply due to better survival of 3E9 cells, because measures of Ag activation were found. CD45RB expression was decreased when activated retinal cells were used as APC, but not to the same level as found with the SPL or CD11c-enriched cells. Because the T cells are Ag experienced, and have a higher FSC even when resting, smaller increases in mean FSC were found. Scatter was increased in cultures containing RetCD45 cells activated with the combination of IFN-γ and anti-CD40.
The expression level of CD4 on T cells is known to be decreased upon activation by Ag/APC (37), cross-linking of TCR (38), bead-bound peptide/MHC (39), and PMA (37). In preliminary studies, we found that expression of CD4 decreases upon activation via the TCR of AE-T cells, providing another measure of T cell activation resulting from APC activity (data not shown). The ratio of CD4\textsuperscript{high} to CD4\textsuperscript{low} T cells decreased in the presence of both activated and control RetCD45 cells and Ag, and even more when conventional APC were used, approaching a ratio of 0.6. Preactivation of RetCD45 cells did not significantly alter the effect on the CD4 ratio of the T cells (Fig. 10B). We interpret this effect on CD4 expression as evidence of TCR ligation on the AE-T cells by Ag-bearing RetCD45 cells, even though other measures of activation, especially recovery of T cells, were less significant.

**Discussion**

The presentation of Ags to T cells in retina is fundamental to local immune responses and immunosurveillance of this tissue, whether for responses to pathogens or for induction and maintenance of peripheral tolerance to retinal self Ags. We found that retinal-derived CD4\textsuperscript{+} cells, even if LPS activated, had little ability to present Ag to naive T cells in a manner that led to expansion of an activated, cytokine-secreting effector population. They were more effective in presentation to Ag-experienced T cells, but much less potent than splenic APC or BrnCD45\textsuperscript{+} cells. This was especially apparent if APC activity was assessed by the recovery of large, activated T cells.

The RetCD45\textsuperscript{+} cells were not neutral with respect to 3E9 T cell activation; there was a moderate ability to limit responses induced by splenic APC, resulting in a dissociation of properties associated with T cell activation. In these examples, CFSE was diluted; FSC, CD69, and CD25 were increased; and CD45RB was decreased, all signs of T cell activation. Regardless of these indicators of activation, the expansion of T cells, in terms of the recovery of live, lymphocyte-sized, V\textsubscript{B10} T cells and IL-2 production, was reduced. A possible explanation for the inhibitory activity is suggested by the presence of the low level of FasL detected on RetCD45 cells, which might induce apoptosis in Fas\textsuperscript{+} T cells. MG in brain have been shown to express a low level of FasL (40, 41), which is greatly up-regulated in vivo by systemic administration of LPS (42), consistent with speculation that MG could exert a regulatory role by inducing apoptosis in infiltrating T cells. In our experiments, we found no evidence for increased apoptosis in the T cells due to the presence of RetCD45 cells, with or without Ag or anti-CD3e activation, in any of our assays. This conclusion is based on analysis of the expression of the active form of caspase-3 (Fig. 7, and data not shown); by the rate of loss of cells from the viable, lymphocyte-sized region of scatter plots (data not shown); or by the appearance of events in a size-gated region corresponding to annexin V\textsuperscript{+}, apoptotic cells by flow cytometry (data not shown).

In cultures in which naive T cells were stimulated in the presence of splenic APC and RetCD45 cells, the smaller number of viable T cells that are recovered also have reduced CFSE levels, indicating that there has been cell division. The time course studies of T cell activation showed that fewer T cells up-regulated CD69 in the presence of RetCD45 cells, but the reduced response was not found to correlate with an increased number of cells with the activated form of caspase-3. The activated cells progress to division, as shown by their CFSE dilution, but without the full yield of viable, activated V\textsubscript{B10}CD4 T cells. Together, the data suggest that fewer T cells are activated in the presence of RetCD45 cells, but that the cells that are activated are capable of at least several divisions. The regulatory activity did not depend on ligation of the TCR by peptide/MHC, because addition of RetCD45 cells from B6 mice also decreased IL-2 production. The activation of AE-T cells by Ag/SPL was not consistently reduced by the inclusion of RetCD45 cells in these cultures. Expression of regulatory activity may be appropriate to the tissue, because Ag presentation that led to a strong, archetypal Th1-like response in retina would be undesirable, given the fragile and nonregenerative nature of the retina and visual axis.

It is clear from many studies that the eye makes a comprehensive effort to regulate intraocular immune responses through the activity of organ-resident cells. To date, studies have concentrated on CD45-negative cells from the epithelial layers of cornea, iris, ciliary body, and retinal pigmented epithelium (RPE). Investigations of the mechanisms have revealed interesting strategies. Pigmented epithelial cells from iris strongly suppressed T cell proliferation and secretion of IFN-\gamma, IL-2, IL-4, and IL-10 in a contact-dependent manner (43). Although the cells made TGF-\beta1 and \beta2 mRNA and IL-10, neutralizing Abs did not reverse the inhibition, nor did addition of exogenous IL-2. Suppression did not appear to involve CD95 or apoptosis. The constitutive expression of CD86 on the iris cells led to inhibition of T cell responses by interaction with CTLA-4 (44).

There are several reports of the immunosuppressive properties of RPE, showing that they possess both soluble and membrane-associated suppressive activities that inhibited T cell activation and proliferation (45). Part of the activity was due to PGE\textsubscript{2} production.
RetCD45 cells were density gradient purified, followed by positive selection with anti-CD45.

The cell-associated activity was removed by trypsin, allowing the RPE to acquire APC activity. Use of human fetal RPE showed that part of the inhibitory activity was due to apoptosis induction that did not act through Fas/FasL interaction, was not blocked by indomethacin, and was not mediated by TGF-β (46). IFN-γ treatment of human fetal RPE increased the potency of the inhibition (47). Human RPE was found to express FasL and induced apoptosis in activated Fas+ T cells, but not resting T cells (48). Studies with an adult RPE cell line, ARPE-19, showed that the inhibition was reversible, and unrelated to interaction with CD2, CD18, CD40, CD40L, CD54, CD58, TCR, or MHC. CD25 expression was reversible, and unrelated to interaction with CD2, CD18, and NO (49). Although RPE cells inhibited T cell proliferation and IL-2 production, IFN-γ-activated RPE was able to stimulate Ag-dependent TNF-α release from a CD4 T cell line. This treatment up-regulated MHC class II on the RPE. Without IFN-γ treatment, the RPE were inhibitory for TNF-α release from the T cells (50).

CD45-negative glial cells of the retina, especially Muller cells, have also been shown to alter T cell activation. Muller cells expressed MHC class II, but did not present Ag to long-term cultured T cells. Instead, the proliferative response and IL-2 production to conventional APC were inhibited by addition of Muller cells (51). Expression of the high affinity IL-2R was also inhibited. Suppression was mediated by a contact-dependent mechanism. However, the production of IL-3 and IFN-γ was not inhibited (52). Mild trypsinization and fixation of the Muller cells after incubation with Ag and IFN-γ revealed the ability to present Ag to a CD4 T cell line (53).

Although the potency of inhibition mediated by RetCD45 cells is much less than that found in other in vitro studies of RPE, our results are consistent with proposals that retinal MG have an immunoregulatory activity that attenuates the severity of inflammatory processes, perhaps helping to spare the retina from damaging responses (19, 54, 55). The TGF-β-rich microenvironment of the retina appears to program the response of resident tissue macrophages such that they are relatively unresponsive to subsequent exposure to inflammatory cytokines, including IFN-γ and TNF-α. Conversely, infiltrating macrophages do respond and produce NO, and are unresponsive to TGF-β. The mechanism of inhibition in our studies is unclear, but the addition of neutralizing Abs for TGF-β1 and β2 did not reverse the inhibition in RetCD45 cell cocultures with splenic APC, or reveal activation in cultures with T cells, Ag, and RetCD45 cells.

Phenotypic characterizations of CD45 + cells in retina reveal evidence for MG and PVC, but cells with characteristics of prototypical DC, immature or mature, have not been found. Bone marrow grafting studies in both CNS and retina support the idea that donor-derived class II MHC-compatible PVC are required to develop autoimmune disease by adoptive transfer of activated T cells (7, 16), which implies a role in Ag presentation. An alternative mechanism of Ag presentation to activated T cells in retina follows from the observation that activated T cells, without specificity for retinal Ag, migrate into retina (6, 22). Entry of activated T cells into retina was accompanied by ED1 + monocytes, and local, minor breakdown of the blood/retinal barrier was found, together with activation of retinal MG (22). These results raise the possibility that retinal APC are recruited, rather than resident, and are consistent with the finding of emigration and reverse transmigration of human monocyte-derived DC (56). In that study, Randolph et al. found that CD16 + monocytes, and local, minor breakdown of the blood/retinal barrier was found, together with activation of retinal MG (22). These results raise the possibility that retinal APC are recruited, rather than resident, and are consistent with the finding of emigration and reverse transmigration of human monocyte-derived DC (56).

Based on these observations, large scale incursions by activated T cells with specificity for retinal Ag, and the proinflammatory cytokines they produce, may tip the balance in favor of a local effector response, as well as activate, T cells (57), and the differentiation resulting from TGF-β1 exposure has been shown to induce some DC to be regulatory or tolerizing (58, 59).
known to generate the regulatory cells of anterior chamber-associated immune deviation (60), an ocular immunoregulatory response. Treatment of immature bone marrow DC with TGF-β and interphotoreceptor retinoid-binding protein, and transfer to naive mice, made them resistant to EAU induction by interphotoreceptor retinoid-binding protein (61). This could also apply to PVC, which exist in the TGF-β-containing environment, and may be programmed to be regulatory as a result.

Comparison of Ag presentation in retina and brain is useful, because retina is a CNS tissue. Studies from our lab (21) and others (20) show the presence of a substantial population of MG in the parenchyma of retina, and there is indirect evidence for PVC in retina (16, 17) based on studies using bone marrow chimeras similar to that reported by Hickey and Kimura (7) in CNS. Isolated rat CNS CD45(high) cells have been reported to be highly functional APC when tested with a T cell line (28). The population enriched for MG was much less able to present Ag. Cultured, IFN-γ-stimulated neonatal MG have been shown to present Ag to naive CD4 T cells (62), but the relationship between these and fresh, adult MG is unclear. Manipulation of these cells may induce a differentiation program unlike that available to the adult MG, and of unknown significance.

An important difference between CNS and retina, with particular relevance to studies of APC activity in retina and brain, is the absence of retinal structures equivalent to the meninges or choroid plexus of the brain that would contaminate tissue isolates. Studies

FIGURE 7. Inclusion of RetCD45 cells reduced the number of T cells that expressed CD69 in response to Ag at 24- and 48-h culture. A. Data from cells size gated to include only enlarged, activated Vβ10+ cells. Note the difference in the scales of the vertical axes between panels on the left side (0–7500 cells) and right side (0–750 cells). B. Data from size-gated cells to include only cells that had not increased FSC or side scatter. Control cultures without Ag showed no evidence of CD69 up-regulation or differences in caspase-3 activation (data not shown).

FIGURE 8. Comparison of the ability of RetCD45 vs BrnCD45 cells to stimulate AE-T cells. Purified RetCD45+ cells are able to induce a modest, Ag-dependent activation of purified, AE-T cells. Cells and supernatants were harvested on day 4. The number of viable Vβ10+ 3E9 T cells was determined by size gating of Vβ10+ cells. FSC, CD25 and CFSE were gated on viable lymphocyte-sized, Vβ10+ T cells. Cultures contained 50 × 10^3 AE-T cells, 25 × 10^3 SPL, and/or 25 × 10^3 RetCD45 or BrnCD45 cells. The AE-T cells were purified by one cycle of negative selection on anti-Ig-coated flasks, followed by density gradient purification, and positive selection with anti-Thy-1.

FIGURE 9. RetCD45 cells induce a small, but significant proliferation in purified AE-T cells, but not IFN-γ production. Cells and supernatants were harvested on day 4. The number of viable Vβ10+ 3E9 T cells was determined by size gating of Vβ10+ cells. FSC, CD25 and CFSE were gated on viable lymphocyte-sized, Vβ10+ T cells. Cultures contained 50 × 10^3 AE-T cells, 25 × 10^3 SPL, and/or 25 × 10^3 RetCD45 cells. The AE-T cells were purified by one cycle of negative selection on anti-Ig-coated flasks, followed by density gradient purification, and positive selection with anti-Thy-1. Anti-TGF-β1 and β2 were used at 5 μg/ml.
of brain show that DC are not found in immunologically quiescent parenchyma, but are recruited after the onset of inflammation (10). DC are found in all layers of the meninges, including the pia mater and the choroid plexus (63, 64) of the CNS, and could be an important source of recruited APC. This localization of DC will lead to contamination of brain preparations with meningeal-derived DC, unless exceptional measures are taken in the dissection of the brain. The result of contamination could be the clear ability to present Ag, as we have found in our assessment of CD45<sup>+</sup> cells from brain that was relatively free of meninges, except for the tightly adherent pia mater. The anatomy of retina avoids this difficulty, and it can be isolated with little contamination, especially when pigmented donors are used, which permits contaminating tissue to be seen and removed. Perfusion before enucleation removes CD45<sup>+</sup> cells from the vasculature. Although the recovery of CD45<sup>+</sup> cells from the retina is more labor intensive than recovery from brain, because of the limited amount of retinal tissue per eye, its ability to be isolated cleanly, and free of DC-containing structures, compensates for the subsequent difficulties that those cells contribute to the interpretation of APC experiments.

It has been postulated that CNS MG are in an undifferentiated state and serve as uncommitted myeloid progenitors of immature DC (65). This is an interesting hypothesis because it is increasingly apparent that immature DC serve an important role in both the induction and maintenance of peripheral tolerance (66). One of the mechanisms by which immature DC induce Ag-specific regulatory T cells is based on DC production of IL-10 (67, 68). MG from both brain and retina produce IL-10, and production is up-regulated in vitro by LPS (69–71). Peripheral tolerance induction by immature DC in vivo does not appear to involve apoptosis of the responding cells, because the response can be rescued by an agonistic anti-CD40 (72). Instead, T regulatory that also produce IL-10 are recruited after the onset of inflammation (74). The expression of MHC class II in vivo induced expression of CD11c on the CD45<sup>high</sup> population of cells (21). Although we have no evidence that these cells are related to retinal MG, the results raise the possibility that a small number of regulatory, immature DC-like cells are present, or can be induced.

Our working hypothesis suggests a multitiered retinal immune response based on the microanatomy of MG and PVC, recruitment of APC from the circulation, and the fact that retinal immune privilege can be overcome by use of strong, Th1-inducing adjuvants. In this scheme, MG are primarily charged with contributing to the immune privilege of the retina. Non-Ag-specific mechanisms include constitutive expression of CD200 on neurons and its receptor on MG (54, 55, 73). The eye maintains a local, TGF-β-rich environment, which appears to alter APC activity in the retina in vivo, as adult RetCD45<sup>+</sup> cells exhibit relatively little response to activators such as IFN-γ, anti-CD40, and LPS treatment in vivo and in vitro (21), or respond in a way that limits their ability to promote inflammation (74). The expression of MHC class II on PVC, and at low levels on MG (21), offers the possibility of Ag-dependent effects on T cells that enter the retina.

Because PVC residing inside the glia limitans appear to have the most intimate contact with the retinal vasculature, the first contact between emigrating activated T cells and CD45<sup>+</sup> cells in retina may be with PVC. The PVC response to this interaction may depend on the potency and nature of the initial encounter with T cells. In a previous study, we found that activation with anti-CD40 in vivo induced expression of CD11c on the CD45<sup>high</sup> population of cells (21). Because T cells that infiltrate are those that are activated, they will express CD40L and produce IFN-γ, if they are Th1-like. Together, these may induce differentiation in PVC that results in APC-like capability. PVC may be the cells responsible for the limited response of the AE-T cells that we found. An initial

### FIGURE 10

Treatment of RetCD45 cells with activators of Ag presentation prepares them to induce a larger response in AE-T cells in the presence of Ag. RetCD45 cells (20 x 10<sup>3</sup>/well), DC (20 x 10<sup>3</sup>/well), and SPL (20 x 10<sup>3</sup>/well) were prepared, as described, dispensed to V-bottom plates, and activated with anti-CD40 (1 μg/ml), LPS (10 μg/ml), IFN-γ (100 U/ml), or the combination of IFN-γ and anti-CD40. The activators were added either 4 days prior, or at the time of addition of AE-T cells and Ag. Cells that were preactivated were washed twice with PBS, and AE-T cells and peptide were added. The AE-T cells were prepared, as described in Materials and Methods. After 4 days coculture, the wells were harvested for flow cytometry. A, Representative results with activated RetCD45 cells and CD11c<sup>+</sup> cells are shown. B, The combined results show that activation of the RetCD45 cells increased the proliferative response of Ag-experienced 3E9 T cells by 2-fold.

<table>
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<tr>
<th>APC treatment</th>
<th>timing</th>
<th>stimulant</th>
<th>APC</th>
<th># of Vβ10&lt;sup&gt;+&lt;/sup&gt; T cells recovered</th>
<th>ratio: CD45&lt;sup&gt;+&lt;/sup&gt;CD45&lt;sup&gt;−&lt;/sup&gt;</th>
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<td>IFN-γ</td>
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<tr>
<td></td>
<td>day -4</td>
<td>α-CD40</td>
<td>IFN-γ</td>
<td>009</td>
<td>090</td>
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<tr>
<td>RetCD45</td>
<td>day 0</td>
<td>α-CD40</td>
<td>None</td>
<td>009</td>
<td>090</td>
</tr>
<tr>
<td>SPL</td>
<td>day 0</td>
<td>IFN-γ &amp; α-CD40</td>
<td>None</td>
<td>009</td>
<td>090</td>
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</tbody>
</table>

The combined results show that activation of the RetCD45 cells increased the proliferative response of Ag-experienced 3E9 T cells by 2-fold.
response resulting from T cell/PVC interaction that is proinflammatory may lead to local degradation of the blood retinal barrier, promoting further emigration and migration of cells from the circulation. Further infiltration of T cells will result in T cell contact with the much larger numbers of MG outside the basal lamina. There, the presence of CD80, low levels of MHC class II, and CD200/CD200R interaction on MG may provide Ag-dependent and independent signals for regulatory responses that could inhibit Th1-like responses. At this time, we cannot distinguish between the activities of the different cell types in our RetCD45 cell preparations. It is almost certain that they have differing and opposing functions with respect to APC activity.

Acknowledgments
We thank Jing Yang for technical assistance, Dr. Linda McLoon for helpful discussions, and Dr. Robert Hendricks for a critique of the manuscript.

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