Published in final edited form as:

Invest Ophthalmol Vis Sci. 2008 January ; 49(1): 282–289. doi:10.1167/iovs.07-0371.

Reactivation of Uveitogenic T Cells by Retinal Astrocytes Derived from Experimental Autoimmune Uveitis-Prone B10RIII Mice

Guomin Jiang, Yan Ke, Deming Sun, Gencheng Han, Henry J. Kaplan, and Hui Shao

From the Department of Ophthalmology and Visual Sciences, Kentucky Lions Eye Center, University of Louisville, Louisville, Kentucky

Abstract

Purpose—To determine the involvement of retinal astrocytes (RACs) in T cell-mediated experimental autoimmune uveitis (EAU).

Methods—Frozen sections of eyes from naive mice or mice with EAU were stained for glial fibrillary acidic protein (GFAP) or major histocompatibility complex (MHC) class II molecules and were examined by confocal microscopy. RACs were isolated and cocultured with interphotoreceptor retinoid-binding protein (IRBP) peptide-specific T cells. The proliferation and cytokine production of responder T cells were determined by [³H]-thymidine incorporation and ELISA, respectively.

Results—The development of intraocular inflammation was associated with increased GFAP-positive cells in the retina. RACs from EAU-prone mice (B10RIII) activated uveitogenic T cells in vitro, enhanced T-cell proliferation and the production of proinflammatory cytokines, and increased the numbers of IL-17⁺ IRBP T cells in the inflamed eye. The interaction between local RACs and IRBP-specific T cells was regulated by a distinct pattern of costimulatory molecules. In addition, the ability of IRBP-specific T cells to interact with RACs was dependent on whether the latter were derived from EAU-prone (B10RIII) or EAU-low susceptible (C57Bl/6) strains of mice.

Conclusions—This study suggests that the RACs in EAU-prone mice contribute to the reactivation of pathogenic T cells in the eye, leading to intraocular inflammation and tissue damage.

The eye, like the central nervous system (CNS), is considered an immunoprivileged site equipped with several regulatory mechanisms that minimize uncontrolled inflammation and immune reactions. The presence of an intact blood-retinal barrier, which limits the passage of T cells into the eye, and the absence or low levels of major histocompatibility complex (MHC) class II and adhesion/costimulatory molecules render the eye an unsuitable site for T-cell priming.^{1,2} Nevertheless, autoreactive T cells activated in peripheral lymphoid organs can cross the blood-retinal barrier,^{3–5} and immune responses associated with intraocular inflammation and damage do frequently occur. It appears that certain resident cells of the eye may play a role in the initiation of intraocular reactivation of these T cells and contribute to an undesired immune response inside the eye. Among these resident cells, retinal glia—microglia, astrocytes, Müller cells, dendritic cells (DCs), and retinal pigment epithelial (RPE) cells are potential candidates because they are able to express MHC class II molecules.^{6–8} However, reactivation of the invading pathogenic T cells within the eye is still poorly understood, and the efficiency of these cell types as local antigen-presenting cells (APCs) that restimulate autoreactive T cells remains unclear.

Corresponding author: Hui Shao, Department of Ophthalmology and Visual Sciences, Kentucky Lions Eye Center, University of Louisville, 301 E. Muhammad Ali Boulevard, Louisville, KY 40202; h0shao01@louisville.edu.

Disclosure: G. Jiang, None; Y. Ke, None; D. Sun, None; G. Han, None; H. J. Kaplan, None; H. Shao, None

Autoreactive CD4⁺ T lymphocytes exhibiting a Th1 phenotype are thought to play a critical role in the pathogenesis of EAU, an animal model of ocular autoimmune disease.^{9,10} Recently, another CD4 effector lineage, Th17, characterized as secreting IL-17, has been described as contributing to the pathogenesis of a number of autoimmune diseases, such as rheumatoid arthritis and experimental autoimmune encephalomyelitis (EAE).^{11–14} However, the data regarding the involvement of this T-cell subset in the pathogenesis of intraocular inflammation is unknown.

In this study, we demonstrated that the development of intraocular inflammation was associated with increased expression of glial fibrillary acidic protein (GFAP) and MHC class II molecules in the retina. Retinal astrocytes (RACs) isolated from the inflamed eye promoted the activation of interphotoreceptor retinoid-binding protein (IRBP) peptide-specific uveitogenic T cells in vitro. We also showed that a subset of the infiltrated T cells was IL-17⁺. In vitro studies have suggested that RACs play a major role in the accumulation and activation of IL-17⁺ autoreactive T cells in the eye. The interaction between local glial cells and IRBP-specific T cells was regulated by a distinct pattern of costimulatory molecules. In addition, the ability of IRBP-specific T cells to interact with RACs differs between those isolated from the EAU-prone B10R/III mouse and the EAU-low susceptible B6 mouse. Our observations indicate an active involvement of RACs in the intraocular immune response in EAU.

Materials and Methods

Animals

Pathogen-free female B10R/III and C57BL/6 mice, purchased from Jackson Laboratory (Bar Harbor, ME), were housed and maintained in the animal facilities of the University of Louisville. All animal studies conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Institutional approval was obtained, and institutional guidelines regarding animal experimentation were followed.

Mice RACs and RPE Cell Isolation

The method to isolate RACs^{15,16} was modified. Briefly, eyes from naive B10R/III or B6 mice (4–6 weeks old) were collected, and the connective tissue was removed. Then the eyes were immersed in Ca²⁺/Mg²⁺-free PBS containing 100 IU/mL penicillin and 100 µg/mL streptomycin on ice for 3 hours. Under a dissection microscope, the anterior segment and vitreous were discarded. The neural retina (for RACs) was incubated with 0.25% trypsin/0.53 mM EDTA at 37°C for 10 minutes. Digestion was terminated by the addition of complete medium (CM; RPMI 1640 medium containing 10% fetal bovine serum, 2 mM glycine [GlutaMAX II; Gibco BRL, Life Technologies, Karlsruhe, Germany], 100 IU/mL penicillin, and 100 µg/mL streptomycin; Sigma), and the cells were centrifuged at 400g for 5 minutes, then dissociated by gentle trituration through a fire-polished Pasteur pipette. After three washes, the cells were resuspended in CM and seeded on poly-D-lysine-coated six-well plates at a density of 5×10^6 cells/well. After incubation for 30 days, the purity of the RACs was greater than 95%, as assessed by staining with primary antibody against GFAP (Sigma) and S-100 (Santa Cruz Biotechnology, Santa Cruz, CA), followed by FITC- or PE-conjugated secondary antibodies (Jackson, Pittsburgh, PA). RACs were used in experiments after culture for 3 to 5 passages.

RPE cell isolation and culture were prepared as described.⁸ More than 95% of the cells in cultures stained positive with FITC-labeled anti-pan keratin antibody (clone PCK-26; Sigma-Aldrich) or RPE65 (Novus, Littleton, CO), indicating that they were virtually all RPE cells.

Coculture of RACs and IRBP Peptide-Specific T Cells

Mitomycin C (MMC)-treated (100 $\mu\text{g}/\text{mL}$ for 1 hour at 37°C) RACs or RPE cells (1×10^5) were incubated with IRBP peptide-specific T cells (3×10^5) in the presence of the immunizing IRBP peptide (10 $\mu\text{g}/\text{mL}$) and in the presence or absence of monoclonal antibodies (mAbs) against costimulatory molecules. The proliferative response of the T cells was measured after 72 hours of culture, and cytokine production by T cells was measured in the culture supernatant after 48 hours of incubation.

Proliferation Assay

T cells from IRBP161-180-immunized B10RIII mice or IRBP1-20-immunized B6 mice were prepared and seeded at 3×10^5 cells/well in 96-well plates, then cultured at 37°C for 72 hours in a total volume of 200 μL medium with or without the immunizing peptides in the presence of irradiated syngeneic spleen APCs (1×10^5), and [^3H]thymidine incorporation during the last 8 hours was assessed using a microplate scintillation counter (Packard Instrument, Meriden, CT).

Cytokine Assay

Supernatants from the cocultures were collected after 48 hours, and IFN- γ , TNF- α , IL-6, IL-10, and IL-17 were measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Actively Induced and Adoptively Transferred Uveitis

For the active induction of disease in B10RIII and B6 mice,¹⁷ the animals were immunized subcutaneously with 100 μL emulsion containing human IRBP161-180 (50 μg) and IRBP1-20 (300 μg), respectively, and 500 μg *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI) in incomplete Freund adjuvant (Sigma), distributed over six spots on the tail base and flank. Concurrently, 0.3 μg pertussis toxin was injected intraperitoneally. For adoptive transfer, recipient animals of the same strain were injected intraperitoneally with 0.2 mL PBS containing 5×10^6 IRBP161-180-specific T cells, prepared as described previously.¹⁷ The clinical course of the disease was assessed by indirect fundus microscopy twice a week and was graded as described previously.¹⁸ The nature of the disease was confirmed histologically.

Treatment with Anti-IL-17 mAb

IRBP161-180-specific T cells from the draining lymph nodes and spleens of donor B10RIII mice immunized with IRBP161-180 were transferred to naive mice, which were injected four times with 5 $\mu\text{g}/\text{eye}$ anti-IL-17 mAb (BioLegend, San Diego, CA) or isotype control antibody at 5-day intervals, starting on the day of transfer (6 mice/group). The animals were examined every 3 days for clinical signs of uveitis by funduscopy, starting at day 8 after transfer.

Pathologic Examination

Inflammation of the eye was confirmed by histopathology. Whole eyes were collected, immersed for 1 hour in 4% phosphate-buffered glutaraldehyde, and transferred to 10% phosphate-buffered formaldehyde until processed. Fixed and dehydrated tissue was embedded in methacrylate, and 5- μm sections were cut through the pupillary-optic nerve plane and stained with hematoxylin and eosin. Presence or absence of disease was evaluated in a masked fashion by examination of six sections cut at different levels of each eye. Severity of EAU was scored on a scale of 0 (no disease) to 4 (maximum disease) in half-point increments, as described previously.¹⁹

Isolation of Cells from Inflamed Eyes

Cells were isolated as described previously.²⁰ At day 23 after T-cell transfer, eyes were collected after PBS perfusion, and a cell suspension was prepared by digestion for 10 minutes at 37°C with collagenase (1 mg/mL) and DNase (100 µg/mL; Sigma) in RPMI 1640 containing 10% FBS, washing, and resuspension in staining buffer (PBS containing 3% FCS and 0.1% sodium azide).

Immunofluorescence Flow Cytometry

Aliquots (1×10^6 cells) were double stained with combinations of FITC- or PE-conjugated mAbs against mouse T-cell receptor, CD4, or CD8 (eBioscience, San Diego, CA). For intracellular cytokine staining, splenic T cells from immunized mice or infiltrated cells from the eye were cultured for 5 hours with 1 µg/mL Brefeldin A, 1 µg/mL ionomycin, and 50 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich). Cells were permeabilized using a kit (Cytofix/Cytoperm Plus; BD PharMingen, San Diego, CA) according to the manufacturer's protocol. Data collection and analysis were performed with a flow cytometer (FACSCalibur; BD PharMingen) and appropriate software (CellQuest; BD PharMingen).

Immunofluorescence Staining for Histology

The frozen section of the eye was air dried for 30 minutes, then fixed in 4% paraformaldehyde for 10 minutes, washed 3 times with PBS, blocked, permeabilized for 1 hour in 2% BSA-0.1% Triton-100, incubated for 1 hour at room temperature with monoclonal anti-mouse GFAP antibody (1:100; Sigma), and for 30 minutes at room temperature with FITC-conjugated second antibody (Jackson, West Grove, PA) and PE-conjugated anti-mouse CD4 (1:100; eBioscience) or PE-conjugated anti-MHC class II molecule antibody (1:100; eBioscience), washed, mounted with DAPI, and viewed on a laser scanning confocal microscope (BX61WI; Olympus, Tokyo, Japan).

Statistical Analysis

The data are expressed as the mean $\pm$ SD. Statistical analysis of the results was performed using Student's *t*-test. $P < 0.05$ was considered significant.

Results

Development of Intraocular Inflammation Is Associated with Increased Expression of GFAP and MHC Class II Molecules in the Retina

To determine the role of local parenchymal cells in ocular inflammation and in the development of uveitis, we performed immunofluorescence staining for GFAP and MHC class II molecules in the eyes of naive mice and mice with uveitis. After the adoptive transfer of IRBP161-180-specific T cells into B10RIII mice, EAU developed between days 8 and 12, rapidly reached maximum severity, and persisted for up to 60 days.^{17,20} Frozen sections of the eye on day 20 after transfer were triple stained using FITC-conjugated anti-GFAP, PE-conjugated anti-MHC class II, or anti-CD4. On confocal microscopy (Fig. 1A), we observed that GFAP expression was greatly increased in the retina of the inflamed eyes of mice with uveitis compared with the eyes of naive mice. Low, but appreciable, levels of MHC class II molecules were coexpressed by some of the GFAP⁺ cells, and there were large numbers of CD4⁺ T cells in the vitreous, with some near GFAP⁺ MHC class II⁺ cells.

To determine whether these GFAP⁺ cells were able to interact with the eye-infiltrating autoreactive T cells, we isolated glial cells from the retinas of naive EAU-prone B10RIII mice and cultured them in vitro for 30 days. The isolated cells were assessed by phase-contrast microscopy and intracellular staining with anti-GFAP,²¹ S-100,²² and vimentin.²³ To exclude

the possibility that the cell preparation was contaminated by RPE cells and macrophages/microglia, we also stained the cells with an RPE cell-specific mAb (RPE 65)²⁴ and an anti-Mac-1/CD11b mAb.²⁵ Double staining with anti-GFAP and S-100 con-firmed that the cell cultures contained 95% to 98% GFAP⁺ S-100⁺ cells (Fig. 1B, upper panel) that were vimentin⁻ (Fig. 1B, lower panel), RPE65, CD45⁻, and MAC-1⁻ (data not shown), suggesting the cultured cells were RACs.

Activation of IRBP-Specific Uveitogenic T Cells In Vitro by RACs Isolated from EAU-Prone Mice

To examine the possible role of RACs in the pathogenesis of intraocular inflammation and the local activation of infiltrated immune cells, we cocultured isolated B10RIII RACs with IRBP161-180-specific T cells and assessed the activation of the T cells. Responder T cells, isolated 13 days earlier from B10RIII mice immunized with IRBP161-180, were seeded into 96-well plates preseeded with isolated RACs pretreated with IFN- γ and MMC and incubated in the presence of IRBP161-180 for 72 hours. For comparison, the same number of T cells was incubated with antigen in the presence of B10RIII splenic APCs or RPE cells. As shown in Figure 2A, proliferation of IRBP161-180-specific T cells was induced with RACs and splenic APCs, but not with RPE cells. Interestingly, RACs from B6 mice had limited ability to induce the proliferation of syngenic IRBP-specific T cells (Fig. 2B).

To determine the ability of RACs to induce cytokine production by responder IRBP161-180-specific T cells, supernatants from the cell cultures described were collected at 48 hours, and their content of IFN- γ , TNF- α , IL-6, and IL-10 was measured by ELISA. Interestingly, the responder T cells produced distinct patterns of cytokines when the reaction occurred in the presence of either RACs or RPE cells. As shown in Figure 2C, the responder T cells produced high amounts of TNF- α and IFN- γ in the presence of RACs but only IL-6 and IL-10 in the presence of RPE cells.

To determine whether the ability of T-cell activation by RACs depended on the expression of costimulatory molecules on their cell surfaces, we compared the expression of MHC class II and costimulatory molecules of B7, CD40, and ICOSL on RACs between B10RIII (susceptible) and B6 (low-susceptible) mice. RACs were cultured with medium or IFN- γ (60 ng/mL) for 72 hours and then stained with antibodies specific for costimulatory molecules. As shown by flow cytometry (Fig. 2D), the costimulatory molecules B7.1, B7.2, and ICOSL had low expression on RACs of B10RIII, but the level of these molecules and MHC class II was increased after stimulation with IFN- γ . In contrast, the costimulatory molecules on RACs of B6 were barely detectable even after stimulation.

Maintenance of IL-17 Secretion Activity of IL-17⁺ IRBP161-180-Specific T Cells In Vitro by RACs

Recent studies have shown that in addition to the previously characterized CD4⁺ T-cell subset (Th1-type) producing inflammatory cytokines (IFN- γ and TNF- α), the T-cell subsets producing IL-17 may play a major role in the pathogenesis of chronic inflammation.^{26,27} To determine whether IL-17⁺ T cells were present in the inflamed eye and whether the number of such T cells correlated with the severity of induced disease, B10RIII mice were adoptively transferred with IRBP161-180-specific T cells, and the eyes were examined for inflammation on day 20 (early disease) or day 50 (late disease). The infiltrated inflammatory cells were collected and intracellularly stained with anti-IL-17 and anti-IFN- γ mAbs. Flow cytometry studies revealed that on day 20, approximately 22% of the eye-infiltrating T cells expressed IL-17 (Fig. 3A), whereas the total number of IL-17⁺ T cells had decreased significantly on day 50. On day 20, the number of IL-17⁺ cells exceeded that of the IFN- γ ⁺ cells, whereas on day 50, the percentage of these two cell types was similar. Interestingly, intravitreal injection of anti-IL-17 (5 μ g/eye,

on days 0, 5, 10, and 15 after transfer) significantly reduced disease severity, and the treated mice showed a significantly decreased number of infiltrating cells in the eye (Fig. 3B).

We hypothesized that activated RACs might have contributed to the increased proportion of intraocular IL-17⁺ T cells. Isolated RACs were cocultured with B10RIII IRBP161-180-specific T cells in the presence of IRBP161-180 for 72 hours, with IL-17 in the culture supernatants measured by ELISA. As shown in Figure 3C, responder T cells cocultured with RACs, but not RPE cells, produced increased amounts of IL-17. We sought to determine whether RACs were capable of maintaining the production of IL-17 by Th17⁺ cells in the eye. We stimulated IRBP161-180-specific T cells from peptide-immunized B10RIII mice with IRBP161-180 in the presence of APCs and IL-23 for 4 days, separated the T-cell blasts, and cocultured them with RACs for another 3 days. We observed that T cells cocultured with RACs produced higher levels of IL-17 than T cells cultured alone or with RPE cells (Fig. 3D).

Requirement for B7.2 and ICOSL in the Proliferation of IRBP-Specific T Cells

To examine the possible mechanisms by which activated RACs promote IRBP-specific T-cell growth and expansion, we investigated the costimulatory molecules involved in the interaction between responder T cells and RACs. Cultured RACs or splenic APCs were cocultured in vitro with responder T cells in the absence or presence of antibodies specific for various costimulatory molecules. As shown in Figure 4A, B10RIII IRBP161-180-specific T cells stimulated by RACs showed significantly in the presence of IRBP161-180, but this effect was significantly blocked by antibodies against B7.2 or inducible T-cell costimulator ligand (ICOSL) and was slightly blocked by antibodies against B7.1 but not by OX40L or 4-1BBL (CD137L). In contrast, the antigen-presenting ability of splenic APCs was mainly affected by anti-B7.1 but only slightly affected by anti-B7.2 (Fig. 4B).

Requirement for Different Costimulatory Molecules in the Induction of Th1 and Th17 Cells

Studies have shown that the differentiation of Th1 and Th17 cells is regulated by multiple factors, including cytokines and costimulatory molecules. We studied whether the production of IFN- γ and IL-17 by uveitogenic T cells was differentially regulated by costimulatory molecules on RACs. Cultured RACs were cocultured with responder T cells in the absence or presence of antibodies specific for various costimulatory molecules. As shown in Figure 5A, IL-17 production of the responder T cells was significantly blocked by mAbs specific for B7.2, ICOSL, and 4-1BBL, whereas the production of IFN- γ was blocked by the antibodies specific for B7.1, B7.2, ICOSL, OX40L, and 4-1BBL (Fig. 5B). Anti-CD40 mAb did not block IFN- γ production but did increase IFN- γ production.

Discussion

Although CNS glial cells have been intensively studied as local APCs during inflammation and autoimmune disease, including EAE and multiple sclerosis (MS), few studies have been performed on the role of retinal glia in intraocular inflammation (i.e., uveitis).^{28–31}

In the brain, it has been shown that perivascular microglia can participate in antigen presentation and the activation of T cells in vivo.³² Whether astrocytes, the more abundant glial cell, also do so is controversial.^{33–37} In inflammatory lesions of EAE and MS, astrocytes express MHC class II molecules, suggesting that these accessory cells may participate in class II-restricted antigen presentation in vivo.^{38–40} Although some investigators have suggested that astrocytes may downregulate T-cell responses,³³ other investigators suggest that microglia, considered more professional APCs,³⁶ can contaminate primary astrocyte cultures and contribute to antigen presentation.³⁵ However, with the use of primary and immortalized pure murine astrocyte cell lines—which avoid individual cell variation and contamination with

microglia—the class II endocytic pathway and antigen-processing of native CNS autoantigens was observed.⁴¹

In this study, we demonstrated that GFAP⁺ RACs may play an active role in the pathogenesis of intraocular inflammation in EAU-prone mice. The marked increase in GFAP expression in the retina suggested that RACs may contribute to T cell-mediated autoimmune inflammation within the eye.

To explore this possibility, we isolated GFAP⁺ RACs and assessed their ability to interact with autoreactive T cells in vitro. Our studies showed that they were fully able to activate autoreactive T cells in the presence of antigen, leading to responder T-cell expansion and the production of proinflammatory cytokines (Fig. 2). However, the increased ability of RACs to promote T-cell proliferation was seen only in the RACs derived from EAU-prone B10RIII mice, not from EAU-low susceptible B6 mice (Fig. 2B), indicating that the ability of RACs to induce uveitogenic T-cell expansion might have been one of the critical factors in the pathogenesis of uveitis. The functional difference between RACs of these two mouse strains may in part be attributed to the expression of costimulatory molecules on their cell surfaces (Fig. 2D).

Two other ocular glial cell types, microglia and Müller cells, have also been shown to promote or inhibit ocular inflammation, depending on the animal strain, the source of glial cells, and the subset of responding T cells. In Lewis rats, an EAU-prone strain, Mac-1⁺ CD45⁺ retinal microglia,⁴² effectively presented antigen to CD4⁺ T cells in vitro.²⁹ The microglia were migrated to the photoreceptor cell layer and generated TNF- α and peroxynitrite in the early phase of EAU, followed by infiltration with macrophages.⁴³ Microglia from EAU low-susceptible C57BL/6 (B6) and B10. A mice had little ability to present antigen to naive T cells and were less potent than splenic APCs or brain CD45⁺ cells in stimulating sensitized T cells.³⁰ Müller cells have also been shown to have a variable effect on T-cell activation. Müller cells expressed MHC class II but did not present antigen to long-term cultured T cells. The proliferative response and IL-2 production of T cells to conventional APCs were inhibited by the addition of Müller cells,⁴⁴ whereas the production of IL-3 and IFN- γ was not.⁴⁵ Furthermore, mild trypsinization and fixation of Müller cells after incubation with antigen and IFN- γ resulted in the ability to present antigen to a CD4⁺ T-cell line.⁴⁶

Activated T cells that enter the retina can also be activated by bone marrow-derived ED1⁺ MHC class II⁺ monocytes⁴⁷ rather than by resident cells. These bone marrow-derived cells can upregulate CD80/86 and MHC class II on encounter with activation factors and can differentiate into DCs in the presence of TGF-1. Depending on the environment, DCs can tolerate or activate the immune response to an antigen. In a recent paper by Xu H et al.,³¹ a novel population of 33D1(+) DCs was identified in the normal mouse retina. The function of these cells remains to be defined, but increased numbers correlated positively with structural abnormalities in the RPE and increased resistance of the mouse strain to EAU.

RACs were also more efficient than RPE cells in activating uveitogenic T cells. RPE cells only partially activated responder T cells, inducing them to produce cytokines without proliferation, which is consistent with our previous observation that rat RPE cells, with or without preactivation by IFN- γ or IFN- γ plus TNF- α , fail to induce either IL-2 production or T-cell proliferation.⁸

RACs from uveitis-prone mice preferentially induced uveitogenic T cells to produce proinflammatory cytokines, such as IFN- γ , TNF- α , and IL-17, whereas RPE cells induced the same T cells to produce IL-6 and IL-10 (Fig. 2). IL-10 production by uveitogenic T cells cultured with RPE cells might therefore have inhibited the proliferation and differentiation of responder T cells into Th1 and Th17 effector cells. The differential regulation of cytokine

production by RACs and RPE cells might have contributed, respectively, to the propagation or inhibition of intraocular inflammation.

Recent studies have shown that IL-17⁺ T cells play a major role in the pathogenesis of autoimmune disease.^{48–50} Our study comparing the phenotype of ocular infiltrating T cells with that of peripheral T (splenic) cells showed a greatly increased number of IL17⁺ T cells among the eye-infiltrating T cells (Fig. 3A). Coculture of RAC with uveitogenic T cells in vitro in the presence of specific antigen resulted in a great increase in IL-17 production by autoreactive T cells (Fig. 3C). It appears that the accumulation and maintenance of increased IL-17⁺ IRBP T cells in the inflamed eye is related to the function of RACs. The fact that intraocular injection of anti-IL-17 antibodies reduced disease severity suggested that IL-17-producing T cells are important in intraocular inflammation. However, many questions remain regarding how the local glia regulate IL17⁺ T cells and the biological function of these cells. It is interesting that the production of either IFN- γ or IL-17 by uveitogenic T cells reactivated by RACs is differentially regulated by costimulatory molecules (Fig. 4).^{11,12,14} Cytokines such as IL-12, IL-23, IL-6, and TGF- β are other critical factors in the differentiation of T cells.^{51,52} Our laboratory is investigating the cytokine environment produced by RACs and infiltrated inflammatory cells.

T-cell activation requires not only antigen but also signals delivered by costimulatory molecules. Our study showed that the costimulatory molecules involved in the interaction between RACs and T cells differ from those involved in the interaction between T cells and professional (splenic) APCs. Although the antigen-presenting capacity of the RACs was significantly blocked by antibodies against B7.2 and ICOSL, that of splenic APCs was mostly blocked by anti-B7.1 (Fig. 4), suggesting that the reactivation of autoreactive T cells after their entry into the inflamed eye differs from their activation in the periphery. In this sense, the blockade of ongoing (or recurrent) autoimmune uveitis may differ from that of acute and monophasic disease and probably requires the blockade of peripheral and intraocular activation of autoreactive T cells given that only newly activated autoreactive T cells are immunologically aggressive.

In conclusion, our data demonstrate that RACs from uveitis-prone mice can act as very efficient APCs in the eye by restimulating Th1 and Th17 cells. Conversely, RPE cells preferentially restimulate Th2 cells. These observations suggested that RAC and RPE cells may play distinct roles in the regulation of the ocular immune response. Our data also suggested that the outcome of the interaction between local parenchyma cells and the invading T cells was diverse, depending on the presence of MHC class II and costimulatory molecules on resident parenchymal cells.^{44–46} Autoreactive T cells entering the eye may be inhibited or may become hyporesponsive on exposure to nonactivated or minimally activated resident cells, which express very low levels of MHC class II and costimulatory molecules. However, invading T cells will be further activated if they are confronted by intraocular parenchymal cells expressing higher levels of MHC class II and costimulatory molecules,^{53,54} leading to the exacerbation of intraocular inflammation. Currently approved therapies for uveitis are directed at the immune system in the peripheral compartment. Modulating the properties of lymphocytes/monocytes destined to access the eye provides an indirect means of affecting the ocular environment. The development of ligands or antagonists that can cross the blood-retinal barrier and can target the functional properties of ocular APCs represents a potentially powerful and more direct approach to uveitis therapy.

Acknowledgements

The authors thank Tom Barkas for editorial assistance.

Supported in part by National Institutes of Health/National Eye Institute Grants EY12974 and EY14599, Vision Research Infrastructure Development Grant R24 EY015636, a Research to Prevent Blindness career development award (HS), and the Commonwealth of Kentucky Research Challenge Trust Fund (HJK).

References

1. Forrester JV, Liversidge J, Dua HS. Regulation of the local immune response by retinal cells. *Curr Eye Res* 1990;9(suppl):183–191. [PubMed: 2200644]
2. Kaestel CG, Lovato P, Odum N, Nissen MH, Ropke C. The immune privilege of the eye: human retinal pigment epithelial cells selectively modulate T-cell activation in vitro. *Curr Eye Res* 2005;30:375–383. [PubMed: 16020268]
3. Dullforce PA, Seitz GW, Garman KL, et al. Antigen-specific accumulation of naive, memory and effector CD4 T cells during anterior uveitis monitored by intravital microscopy. *Cell Immunol* 2006;239:49–60. [PubMed: 16712823]
4. Thureau SR, Mempel TR, Flugel A, et al. The fate of autoreactive, GFP+ T cells in rat models of uveitis analyzed by intravital fluorescence microscopy and FACS. *Int Immunol* 2004;16:1573–1582. [PubMed: 15351788]
5. Crane IJ, Xu H, Wallace C, et al. Involvement of CCR5 in the passage of Th1-type cells across the blood-retina barrier in experimental autoimmune uveitis. *J Leukoc Biol* 2006;79:435–443. [PubMed: 16365158]
6. Zhang J, Wu GS, Ishimoto S, Pararajasegaram G, Rao NA. Expression of major histocompatibility complex molecules in rodent retina: immunohistochemical study. *Invest Ophthalmol Vis Sci* 1997;38:1848–1857. [PubMed: 9286275]
7. Liversidge J, Sewell HF, Thomson AW, Forrester JV. Lymphokine-induced MHC class II antigen expression on cultured retinal pigment epithelial cells and the influence of cyclosporin A. *Immunology* 1988;63:313–317. [PubMed: 3127329]
8. Sun D, Enzmann V, Lei S, Sun SL, Kaplan HJ, Shao H. Retinal pigment epithelial cells activate uveitogenic T cells when they express high levels of MHC class II molecules, but inhibit T cell activation when they express restricted levels. *J Neuroimmunol* 2003;144:1–8. [PubMed: 14597092]
9. Atalla L, Linker-Israeli M, Steinman L, Rao NA. Inhibition of autoimmune uveitis by anti-CD4 antibody. *Invest Ophthalmol Vis Sci* 1990;31:1264–1270. [PubMed: 1973156]
10. Xu H, Rizzo LV, Silver PB, Caspi RR. Uveitogenicity is associated with a Th1-like lymphokine profile: cytokine-dependent modulation of early and committed effector T cells in experimental autoimmune uveitis. *Cell Immunol* 1997;178:69–78. [PubMed: 9184700]
11. Dong C. Diversification of T-helper-cell lineages: finding the family root of IL-17-producing cells. *Nat Rev Immunol* 2006;6:329–334. [PubMed: 16557264]
12. Harrington LE, Hatton RD, Mangan PR, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 2005;6:1123–1132. [PubMed: 16200070]
13. Mathur AN, Chang HC, Zisoulis DG, et al. T-bet is a critical determinant in the instability of the IL-17-secreting T-helper phenotype. *Blood* 2006;108:1595–1601. [PubMed: 16670261]
14. Park H, Li Z, Yang XO, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 2005;6:1133–1141. [PubMed: 16200068]
15. Scheef E, Wang S, Sorenson CM, Sheibani N. Isolation and characterization of murine retinal astrocytes. *Mol Vis* 2005;11:613–624. [PubMed: 16148882]
16. Malgorzata GI, Raap M, Weick M, et al. The activation of IL-8 receptors in cultured guinea pig Muller glial cells is modified by signals from retinal pigment epithelium. *J Neuroimmunol* 2005;161:49–60. [PubMed: 15748943]
17. Shao H, Fu Y, Liao T, et al. Anti-CD137 mAb treatment inhibits experimental autoimmune uveitis by limiting expansion and increasing apoptotic death of uveitogenic T cells. *Invest Ophthalmol Vis Sci* 2005;46:596–603. [PubMed: 15671287]
18. Thureau SR, Chan CC, Nussenblatt RB, Caspi RR. Oral tolerance in a murine model of relapsing experimental autoimmune uveoretinitis (EAU) induction of protective tolerance in primed animals. *Clin Exp Immunol* 1997;109:370–376. [PubMed: 9276535]

19. Shao H, Liao T, Ke Y, Shi H, Kaplan HJ, Sun D. Severe chronic experimental autoimmune uveitis (EAU) of the C57BL/6 mouse induced by adoptive transfer of IRBP1-20-specific T cells. *Exp Eye Res* 2006;82:323–331. [PubMed: 16125173]
20. Liao T, Ke Y, Shao WH, et al. Blockade of the interaction of leukotriene b4 with its receptor prevents development of autoimmune uveitis. *Invest Ophthalmol Vis Sci* 2006;47:1543–1549. [PubMed: 16565390]
21. Bignami A, Eng LF, Dahl D, Uyedo CT. Localization of the glial fibrillary acidic protein in astrocytes in immunofluorescence. *Brain Res* 1972;43:429–435. [PubMed: 4559710]
22. Chan-Ling T, McLeod DS, Hughes S, et al. Astrocyte-endothelial cell relationships during human retinal vascular development. *Invest Ophthalmol Vis Sci* 2004;45:2020–2032. [PubMed: 15161871]
23. Nakazawa T, Takeda M, Lewis GP, et al. Attenuated glial reactions and photoreceptor degeneration after retinal detachment in mice deficient in glial fibrillary acidic protein and vimentin. *Invest Ophthalmol Vis Sci* 2007;48:2760–2768. [PubMed: 17525210]
24. Boulanger A, Liu S, Henningsgaard AA, Yu S, Redmond TM. The upstream region of the *Rpe65* gene confers retinal pigment epithelium-specific expression in vivo and in vitro and contains critical octamer and E-box binding sites. *J Biol Chem* 2000;275:31274–31282. [PubMed: 10896939]
25. de Groot CJ, Hupples W, Sminia T, Kraal G, Dijkstra CD. Determination of the origin and nature of brain macrophages and microglial cells in mouse central nervous system, using non-radioactive in situ hybridization and immunoperoxidase techniques. *Glia* 1992;6:301–309. [PubMed: 1281462]
26. Kolls JK, Linden A. Interleukin-17 family members and inflammation. *Immunity* 2004;21:467–476. [PubMed: 15485625]
27. Stumhofer JS, Laurence A, Wilson EH, et al. Interleukin 27 negatively regulates the development of interleukin 17-producing T helper cells during chronic inflammation of the central nervous system. *Nat Immunol* 2006;7:937–945. [PubMed: 16906166]
28. Chen L, Yang P, Kijlstra A. Distribution, markers, and functions of retinal microglia. *Ocul Immunol Inflamm* 2002;10:27–39. [PubMed: 12461701]
29. Matsubara T, Pararajasegaram G, Wu GS, Rao NA. Retinal microglia differentially express phenotypic markers of antigen-presenting cells in vitro. *Invest Ophthalmol Vis Sci* 1999;40:3186–3193. [PubMed: 10586941]
30. Gregerson DS, Sam TN, McPherson SW. The antigen-presenting activity of fresh, adult parenchymal microglia and perivascular cells from retina. *J Immunol* 2004;172:6587–6597. [PubMed: 15153473]
31. Xu H, Dawson R, Forrester JV, Liversidge J. Identification of novel dendritic cell populations in normal mouse retina. *Invest Ophthalmol Vis Sci* 2007;48:1701–1710. [PubMed: 17389502]
32. Hickey WF, Kimura H. Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vivo. *Science* 1988;239:290–292. [PubMed: 3276004]
33. Meinl E, Aloisi F, Ertl B, et al. Multiple sclerosis immunomodulatory effects of human astrocytes on T cells. *Brain* 1994;117:1323–1332. [PubMed: 7820569]
34. Frei K, Lins H, Schwerdel C, Fontana A. Antigen presentation in the central nervous system: the inhibitory effect of IL-10 on MHC class II expression and production of cytokines depends on the inducing signals and the type of cell analyzed. *J Immunol* 1994;152:2720–2728. [PubMed: 8144879]
35. Nikcevic KM, Gordon KB, Tan L, et al. IFN-gamma-activated primary murine astrocytes express B7 costimulatory molecules and prime naive antigen-specific T cells. *J Immunol* 1997;158:614–621. [PubMed: 8992975]
36. Matsumoto Y, Ohmori K, Fujiwara M. Immune regulation by brain cells in the central nervous system: microglia but not astrocytes present myelin basic protein to encephalitogenic T cells under in vivo-mimicking conditions. *Immunology* 1992;76:209–216. [PubMed: 1378815]
37. Williams K Jr, Ulvestad E, Cragg L, Blain M, Antel JP. Induction of primary T cell responses by human glial cells. *J Neurosci Res* 1993;36:382–390. [PubMed: 7903705]
38. Traugott U, Scheinberg LC, Raine CS. On the presence of Ia-positive endothelial cells and astrocytes in multiple sclerosis lesions and its relevance to antigen presentation. *J Neuroimmunol* 1985;8:1–14. [PubMed: 3882754]
39. Sakai K, Tabira T, Endoh M, Steinman L. Ia expression in chronic relapsing experimental allergic encephalomyelitis induced by long-term cultured T cell lines in mice. *Lab Invest* 1986;54:345–352. [PubMed: 2419666]

40. Traugott U. Detailed analysis of early immunopathologic events during lesion formation in acute experimental autoimmune encephalomyelitis. *Cell Immunol* 1989;119:114–129. [PubMed: 2784075]
41. Soos JM, Morrow J, Ashley TA, Szente BE, Bikoff EK, Zamvil SS. Astrocytes express elements of the class II endocytic pathway and process central nervous system autoantigen for presentation to encephalitogenic T cells. *J Immunol* 1998;161:5959–5966. [PubMed: 9834077]
42. Penfold PL, Provis JM, Liew SC. Human retinal microglia express phenotypic characteristics in common with dendritic antigen-presenting cells. *J Neuroimmunol* 1993;45:183–191. [PubMed: 8392519]
43. Rao NA, Kimoto T, Zamir E, et al. Pathogenic role of retinal microglia in experimental uveoretinitis. *Invest Ophthalmol Vis Sci* 2003;44:22–31. [PubMed: 12506051]
44. Caspi RR, Roberge FG, Nussenblatt RB. Organ-resident, nonlymphoid cells suppress proliferation of autoimmune T-helper lymphocytes. *Science* 1987;237:1029–1032. [PubMed: 2956685]
45. Caspi RR, Roberge FG. Glial cells as suppressor cells: characterization of the inhibitory function. *J Autoimmun* 1989;2:709–722. [PubMed: 2529864]
46. Roberge FG, Caspi RR, Nussenblatt RB. Glial retinal Muller cells produce IL-1 activity and have a dual effect on autoimmune T helper lymphocytes: antigen presentation manifested after removal of suppressive activity. *J Immunol* 1988;140:2193–2196. [PubMed: 2965182]
47. Hu P, Pollard JD, Chan-Ling T. Breakdown of the blood-retinal barrier induced by activated T cells of nonneural specificity. *Am J Pathol* 2000;156:1139–1149. [PubMed: 10751337]
48. Kikly K, Liu L, Na S, Sedgwick JD. The IL-23/Th(17) axis: therapeutic targets for autoimmune inflammation. *Curr Opin Immunol* 2006;18:670–675. [PubMed: 17010592]
49. Lohr J, Knoechel B, Wang JJ, Villarino AV, Abbas AK. Role of IL-17 and regulatory T lymphocytes in a systemic autoimmune disease. *J Exp Med* 2006;203:2785–2791. [PubMed: 17130300]
50. Yen D, Cheung J, Scheerens H, et al. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Invest* 2006;116:1310–1316. [PubMed: 16670770]
51. Goriely S, Goldman M. The interleukin-12 family: new players in transplantation immunity? *Am J Transplant* 2007;7:278–284. [PubMed: 17229073]
52. Yang XO, Panopoulos AD, Nurieva R, et al. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J Biol Chem* 2007;282:9358–9363. [PubMed: 17277312]
53. el-Asrar AM, Maimone D, Morse PH, Lascola C, Reder AT. Interferon-gamma and tumour necrosis factor induce expression of major histocompatibility complex antigen on rat retinal astrocytes. *Br J Ophthalmol* 1991;75:473–475. [PubMed: 1908315]
54. Akaishi K, Ishiguro S, Durlu YK, Tamai M. Quantitative analysis of major histocompatibility complex class II-positive cells in posterior segment of Royal College of Surgeons rat eyes. *Jpn J Ophthalmol* 1998;42:357–362. [PubMed: 9822962]

Figure 1.

Increased expression of GFAP and MHC class II molecules in the retina during intraocular inflammation. (A) Frozen section of the eye prepared from a naïve or B10RIII mouse adoptively transferred with IRBP161-180-specific T cells 20 days earlier (active disease) and stained with FITC-conjugated isotype control antibody or FITC-conjugated anti-GFAP mAb (green), PE-conjugated anti-MHC class II or anti-CD4 mAb (red), and DAPI (blue) and analyzed using confocal microscopy. (B) Characterization of retinal astrocyte cell cultures. RACs from B10RIII mice were prepared as described in Materials and Methods and were used between passages 3 and 5. The cells were stained with mAbs against GFAP and S-100 (*upper panel*) or GFAP and vimentin (*lower panel*) and were examined by immunofluorescence microscopy at $\times 40$ magnification.

Figure 2.

RACs strongly promote specific uveitogenic T-cell proliferation and differentiation into Th1 cells. **(A)** RACs are much more efficient than RPE cells in presenting uveitogenic peptide, leading to the proliferation of IRBP peptide-specific T cells in EAU-prone mice. T cells (3×10^5) from in vivo peptide-primed B10RIII mice were cultured with 1×10^5 of three types of syngeneic APCs, (MMC-treated RACs, MMC-treated RPE cells, irradiated spleen cells) in the presence of $10 \mu\text{g/mL}$ IRBP161-180. Proliferation was measured by the incorporation of [^3H]-thymidine ($0.5\text{--}1 \mu\text{Ci/well}$) during the last 8 hours of a 72-hour incubation period. Data shown are from 1 of 4 representative experiments. **(B)** RACs from mice with low susceptibility to EAU fail to induce uveitogenic T-cell proliferation. Irradiated splenic APCs or MMC-treated RACs from naive C57BL/6 mice were incubated with T cells from IRBP1-20-immunized B6 mice and IRBP1-20, and proliferation was measured as described in **(A)**. Results shown are representative of those for three separate experiments. **(C)** RACs efficiently induced IRBP-specific T cells to produce proinflammatory cytokines. The experimental paradigm was as described in **(A)**, but, after 48 hours, the supernatants from triplicate cultures were pooled, and cytokines were measured by ELISA. Values are the mean $\pm$ SE of three individual experiments. **(D)** MHC class II and costimulatory molecule expression on B10RIII and B6 RACs. RACs (5

$\times 10^5$) were incubated in medium alone or medium containing IFN- γ (60 ng/mL); the trypsinized cells were stained with anti-MHC class II, B7.1, B7.2, CD40, and ICOSL mAbs and were analyzed by flow cytometry. The x -axis represents fluorescence intensity on an arbitrary log scale, and the y -axis represents cell number. Negative control samples were stained with an isotype-matched control mouse IgG antibody.

Figure 3.

IL-17-producing T cells were critical inflammatory cells in the development of uveitis, and RACs were able to induce and maintain the production of IL-17 by IL-17-producing uveitogenic T cells. (A) IL-17⁺ T cells are present in the eye in uveitis. B10RIII mice were adoptively transferred with activated IRBP161-180-specific T cells (3×10^6 /mouse). On days 20 (early disease) or 50 (late disease), spleen cells or eye infiltrating cells (3 mice/group) were collected and restimulated with PMA and ionomycin for 5 hours, and intracellular detection of IL-17 and IFN- γ was performed. Numbers indicate the percentages of positive cells in that region. The experiments were repeated at least twice, with similar results. (B) Local injection of anti-IL-17 mAb reduced the severity of disease. B10RIII mice were adoptively transferred with IRBP161-180-specific T cells. One group received isotype control mAbs, and the second received anti-IL-17 mAbs administered intravitreally at a dose of 5 μ g/eye on days 0, 5, 10, and 15 after T-cell transfer; the course of disease severity was documented by funduscopy and histology. (C) IL-17 production by T cells is induced by RACs. Splenic T cells from IRBP161-180-immunized B10RIII mice were stimulated with IRBP161-180 peptide and RACs for 72 hours in vitro, and then IL-17 in the supernatant was measured by ELISA. Results shown are the mean $\pm$ SE of the results of three separate experiments. (D) IL-17-producing T cells were maintained by RACs. Splenic T cells from IRBP161-180-immunized B10RIII mice were stimulated with IRBP161-180 and irradiated syngeneic splenic APCs for 72 hours in vitro. Activated T cells were isolated by gradient centrifugation on sterile, endotoxintested solution and incubated alone or with B10RIII RACs or RPE cells for 3 days, and IL-17 in the supernatant was measured by ELISA.

Figure 4.

RACs required unique costimulatory molecules for antigen presentation to uveitogenic T cells. T cells (3×10^5) from IRBP161-180-immunized B10RIII mice were incubated with irradiated splenic APCs (**A**) or MMC-treated RACs from naive B10RIII mice (**B**) (1×10^5) and IRBP161-180 ($10 \mu\text{g/mL}$) in the presence ($10 \mu\text{g/mL}$) or absence of mAbs against costimulatory molecules. T-cell proliferation was measured at 72 hours by [^3H]-thymidine uptake. Results shown are the average of triplicate wells in 1 of 5 representative experiments. Statistically significant differences from the control values are shown * $P < 0.05$, ** $P < 0.01$).

Figure 5.

Different costimulatory molecules are required for RACs to induce IFN- γ or IL-17 production by splenic T cells. Splenic T cells isolated from B10RIII mice immunized with IRBP161-180 were restimulated with IRBP161-180 in the presence of RACs for 48 hours in vitro in the presence or absence of various mAbs against costimulatory molecules. IL-17 (A) and IFN- γ (B) levels in the supernatants were measured by specific ELISA kits. Data are representative of at least three independent experiments with similar results. Statistically significant differences from the control values are shown (* $P < 0.05$, ** $P < 0.01$).