Retinal Laser Burn Disrupts Immune Privilege in the Eye

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Immune privilege allows for the immune protection of the eye in the absence of inflammation. Very few events are capable of overcoming the immune-privileged mechanisms in the eye. In this study, we report that retinal laser burn (RLB) abrogates immune privilege in both the burned and nonburned eye. As early as 6 hours after RLB, and as late as 56 days after RLB, antigen inoculation into the anterior chamber of the burned eye failed to induce peripheral tolerance. After RLB, aqueous humor samples harvested from nontreated eyes but not from either the burned or the contralateral eye, down-regulated the expression of CD40 and up-regulated interleukin-10 mRNA in peritoneal exudate cells, and converted peritoneal exudate cells into tolerogenic antigen-presenting cells (APCs). Unlike F4/80⁺ APCs from nontreated mice, F4/80⁺ APCs from RLB mice were unable to transfer tolerance after anterior chamber inoculation of antigen into naïve mice. The increased use of lasers in both the industrial and medical fields raises the risk of RLB-associated loss of immune regulation and an increased risk of immune inflammation in the eye. (*Am J Pathol* 2009, 174:414–422; DOI: 10.2353/ajpath.2009.080766)

Since light amplification by stimulated emission of radiation (laser) was first demonstrated in 1961,¹ the use of lasers in the research, industrial, and military fields, and a corresponding number of occupational eye accidents, has increased.^{2,3} Many such ocular injuries lead to retinal destruction with massive photoreceptor loss and severe visual impairment.⁴ Similarly, visual impairment is often observed after therapeutic retinal photocoagulation treatment, a common procedure in clinical practice. Ophthalmic laser treatment is the standard therapy for many sight-impairing retinal disorders, such as age-related macular degeneration, and its advanced stages of the disease, including choroidal neovascular elements.⁵ However, the laser-

treated eyes are often complicated by the immediate visual impairment that is caused by the unavoidable laser-induced destruction of the normal tissue adjacent to the lesion.⁶

The eye is endowed with immune regulatory mechanisms (immune privilege)⁷ that protect the delicate ocular tissues from inflammatory damage. Inflammation can interfere with the visual pathways and in some cases lead to blindness. Immune privilege is actually thought to be an evolutionary compromise to preserve the delicate microanatomy of the eye while maintaining ocular immune responses. Intraocular injection of exogenous antigens induces a stereotypic alteration in the systemic immune response termed anterior chamber-associated immune deviation (ACAID).⁸ ACAID results in the activation of a modified antibody response with T-cell-mediated suppression of Th1⁹ and Th2 responses.¹⁰

The importance of inflammation in laser injuries has been suspected but not studied in detail. There has been no rigorous identification of either the inflammatory cells that might produce damage to the retina after laser burn or the cytokines released by these cells that might contribute to retinal destruction. Current medical treatment for laser injuries is systemic administration of anti-inflammatory drugs, typically corticosteroids. The corticosteroid treatment is believed to limit retinal injury, reduce visual loss, and increase recovery.^{11,12}

Understanding the extent of the inflammatory response is a crucial step in diminishing or limiting the extent of the laser-induced secondary retinal inflammatory damage. Here we examine the postulate that laser burn to the back of the eye affects immune privilege throughout the eye and alters critical mechanisms in the eye of immune regulation toward inflammation.

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Materials and Methods

Mice

C57BL/6 (B6) mice were purchased from Taconic Farms (Germantown, NY). Female, 8- to 12-week-old mice were used in all experiments. EGFP transgenic female mice (B6 background)¹³ were purchased from The Jackson Laboratory (Bar Harbor, ME) and also bred in our animal colony. All animals were treated humanely and in accordance with the Schepens Eye Research Institute Animal Care and Use Committee and the National Institutes of Health guidelines.

Laser Burn Model

Mice were anesthetized with ketamine (62.5 mg/kg) and xylazine (12.5 mg/kg) and pupils were dilated with 1% tropicamide. A diode laser (wave length 810 nm, diameter 200 nm, power 50 mW, duration time 50 ms) was delivered to the retina through a slit lamp microscope. The posterior pole of the retina was thus burned while a hand-held cover slide was used as a contact lens. Four spots (200 nm in diameter) were burned at the 9 o'clock position of the retina in the right eye of B6 mice. Only lesions in which a subretinal bubble or focal serous detachment of the retina developed were used for the experiments.

Histological Analysis

Eyes were enucleated at various time points (1, 4, 7, 14, 21, 56 days) after laser burn and fixed in 10% formalin for 24 hours. Tissue samples were dehydrated and embedded in paraffin. Sections (6 μ m) were then prepared and subsequently stained with hematoxylin and eosin (H&E) solution to assess the histology of the laser lesion.

Retinal Pigment Epithelium (RPE)-Choroid-Scleral Flatmount

C57BL/6 mice were intravenously injected with 0.2 ml of phosphate-buffered saline (PBS) containing peritoneal exudate cells (PECs, 10^6) harvested from EGFP transgenic mice. One day later, RLB was performed. RPE-choroid-scleral flatmounts were prepared after 24 hours and evaluated. The eyes were fixed in 4% paraformaldehyde, Sigma Aldrich (3 hours). The anterior segment and neurosensory retina were removed, and peripheral choroid and sclera were dissected and flat-mounted on microscope slides for examination with a fluorescence microscope.¹⁴ To test for blood-ocular barrier leakage, mice were deeply anesthetized 24 hours after RLB and perfused through the left ventricle with fluorescein-dextran (0.03 ml/g body weight 50 mg/ml solution of 2×10^6 molecular weight; Sigma, St. Louis, MO).

ACAID Induction and Assay for Delayed Hypersensitivity (DH)

ACAID was induced in mice by inoculating ovalbumin (OVA) [50 μ g/2 μ l in Hanks' balanced salt solution (HBSS), Sigma Aldrich] into the anterior chamber 7 days before sensitizing, subcutaneously OVA (100 μ g/ml in HBSS, 50 μ l) emulsified in complete Freund's adjuvant (CFA) Sigma Aldrich (50 μ l). One week later mice were tested for the development of DH by an intradermal inoculation of OVA-pulsed PECs (2×10^5 cells in 10 μ l of HBSS) into the right ear pinnae. Ear swelling was measured 24 hours later with an engineer's micrometer (Mitutoyo, Paramus, NJ). Laser was performed 1, 4, 7, 14, 21, and 56 days before ACAID induction and the DH was tested by measurement of ear thickness.

Preparation of PECs

PECs were obtained from peritoneal washes of mice 3 days after they received an intraperitoneal inoculation of 2.5 ml of 3% aged thioglycolate solution (Difco, Detroit, MI). Nonadherent cells were removed from the cultures after 18 hours by three washes, and the remaining adherent cells were incubated for 1.5 hours in cold PBS (4°C) followed by vigorous pipetting to collect the cells.

Preparation of Tolerogenic Antigen-Presenting Cells (APCs)

PECs (2×10^5) were then cultured with OVA Sigma Aldrich (5 mg/ml) with or without aqueous humor (AqH) or OVA plus transforming growth factor (TGF)- β (R&D Systems, Minneapolis, MN) in 96-well culture plates. Serum-free RPMI 1640 (Cambrex Bio Science, Walkersville, MD) supplemented with 10 mmol/L HEPES, 0.1 mmol/L non-essential amino acids, 1 mmol/L sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin (all from Invitrogen, Carlsbad, CA), 0.1% bovine serum albumin (Sigma Aldrich), and ITS culture supplement (BD Biosciences, San Jose, CA) were used as media.

Flow Cytometry

PECs were analyzed by flow cytometry. The following antibodies were purchased from BD Pharmingen (San Diego, CA): Fc block, phycoerythrin (PE)-CD40, PE-anti-rat IgG2a, fluorescein isothiocyanate-F4/80, and PE-Cd11b. Staining was performed in the presence of saturating concentration of Fc block (blocks FcR γ II/III) and PE-conjugated anti-CD40 mAb and isotype control of PE-conjugated anti-rat IgG2a. Stained cells were analyzed on an EPICS XL flow cytometry (Beckman Coulter, Miami, FL).

Aqueous Humor (AqH) Collection and Analysis

AqH was obtained from eyes of mice immediately after their euthanasia. AqH samples from panels of at least five

mice (15 eyes, 3 to 5 μ l/eye) at each time point were pooled and centrifuged at $200 \times g$ for 3 minutes to sediment cells; the cell-free supernatants were cultured with 2×10^5 PECs.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was isolated from PECs. One hundred ng of total RNA was reverse transcribed and amplified using the Access RT-PCR system (Promega, Madison, WI) according to the manufacturer's specifications. RT-PCR products were resolved by electrophoresis in a 1.5% agarose gel containing Gel Star nucleic acid stain (Cambrex). The bands were visualized and the gels were photographed using a Molecular FX Imaging station and GelDoc (both from Bio-Rad, Hercules, CA). The primers used were as follows: murine interleukin (IL)-10, sense 5'-ACCTGGTAGAAGTGATGCCCCAGGCA-3', anti-sense 5'-CTATGCAGTTGATGAAGATGTCAAA-3'; murine β -actin, sense 5'-GTGGGCCGCTCTAGGCACCAA-3', antisense 5'-CTCTTTGATGTCACGCACGATTTC-3'.

Adherent Cells Transfer to Naïve Mice

Seven days after anterior chamber inoculation of OVA, mice were euthanized. Spleens were removed from mice

and placed in HBSS. Single cell suspension was prepared by gently tapping minced spleen through a wire mesh screen. Cells were washed and resuspended in HBSS for counting and then plated at 2×10^6 cells/ml and incubated for 90 minutes at 37°C. Nonadherent cells were removed by gentle washing with PBS. To release adherent cells ice-cold PBS was added for 10 minutes before scraping the plates. Dissociated adherent cells were counted and resuspended to 1×10^6 cells/100 μ l.

Statistics

Data were subjected to analysis by analysis of variance and Scheffé's test. The data are presented as mean $\pm$ SEM. An asterisk indicates a statistically significant difference between two groups. A value of $P \leq 0.05$ was considered significant.

Results

Damage Caused by the Retinal Laser Burn (RLB)

To demonstrate that the RLB causes a break in the blood ocular barrier, we injected green cells from EGFP mice into mice before delivering RLB in four separate spots to

Figure 1. Photomicrographs of RPE choroid-scleral flat mounts. **A:** Photomicrograph of infiltrating green cells. GFP-PECs were injected intravenously into C57BL/6 mice 1 day before RLB treatment (top right and left, bottom right), or not (bottom left). Eyes were enucleated 1 day after RLB and the RPE-choroid-scleral flat mount was examined by fluorescence microscope. Infiltrating GFP-PECs are seen as green spots within the areas burned by the laser. Magnification as indicated in the photomicrograph. **B:** Photomicrograph of fluorescein leakage. Mice were injected intravenously with fluorescein-dextran on the same day and before receiving RLB. The eyes were excised and choroidal flat mounts were examined by fluorescence microscope. The prominent fluorescein leakage (arrows) indicates the breakage of Bruch's membrane and the blood-ocular barrier. **C:** Photomicrograph of paraffin-fixed slides stained with H&E of the retina of nontreated (WT) mice or RLB-treated mice. One day after laser treatment the Bruch's membrane [choroid (CH)], retinal pigment epithelium (RPE), outer nuclear layer (ONL), and photoreceptor segment were damaged. RPEs are nonpigmented and appear to migrate toward the ONL (arrow) at 7 to 56 days after RLB. Small lumens (arrow) are evident in the subretinal space and choroidal vessels are dilated at 1 to 56 days after RLB. INL, internal nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium; CH, choroid. Original magnifications, $\times 400$ (C).

one retina. Each laser pulse was from a diode laser. Four days before RLB, we inoculated the thioglycolate-induced PECs from EGFP C57BL/6 mice. One day after the RLB, the mice were euthanized and RPE-choroid-scleral flat mounts were prepared for examination by fluorescence microscope. Unlike the flat, mount preparations from the nontreated control mouse eye or the contralateral eye, the retina preparations from the RLB eye showed green cells within the boundaries of the burn (Figure 1A). These data are consistent with previous studies that showed F4/80-positive GFP-labeled cells infiltrated the retina in a model of laser-induced choroidal neovascular elements.^{15,16} In an alternative experiment, mice were injected with fluorescein-dextran 1 day after RLB. When the RPE-choroid-scleral flat mounts of the RLB eye were examined by fluorescence microscopy, three brightly fluorescing areas (indicating the fluorescein leaked in the choroid) were observed (Figure 1B).

Other experiments examined histological changes in the tissue throughout time after RLB for evidence of RLB-induced damage. Examination of the H&E-stained section showed destruction of Bruch's membrane and RPE (Figure 1C). Although there was damage to the outer nuclear layer (ONL) and the photoreceptor segment, the inner retina was intact. The choroidal vessels are dilated suggesting an active inflammatory response was occurring. A few RPE were seen in the ONL; other RPE appeared to have lost their pigment (day 7 to day 21). Fifty-six days after laser treatment, only a few small holes remained in the subretinal space and most lesions examined appeared closed (healed) and were surrounded by RPE cells.

RLB Interferes with the Induction of ACAID

Because interference with the blood-ocular barrier may allow for unwanted inflammation, we tested the postulate that a laser burn to the retina disrupted immune privilege throughout the eye. In these experiments, groups of mice received laser burn, or not, and at various time points after, we assessed the immune-privileged status in their eyes by inducing ACAID. We observed that as early as 6 hours and as late as 56 days after RLB, the ability to induce ACAID in the eye that received the RLB was impaired (Figure 2). Thus, the induction of an inflammatory response in the back of the eye altered the immune privilege in the anterior segment of the eye and potentially throughout the eye for an extended period.

RLB Interferes with ACAID in Both RLB Eye and the Contralateral Eye

In many models of RLB, the contralateral eye is used as a control eye. To test if RLB had an effect on the contralateral eye, mice received RLB in the right eye, but the susceptibility to ACAID induction was tested by injecting antigen (anterior chamber) in the contralateral eye (left eye). Surprisingly, we were unable to induce ACAID through the contralateral eye (Figure 3). Thus, RLB to one

Figure 2. DH assay for induction of ACAID. OVA was inoculated subcutaneously into C57BL/6 mice and a week later DH was determined after ear challenge in mice that received different treatments (indicated under abscissa and each bar). Laser = RLB treatment; a.c. = OVA inoculated into the anterior chamber; s.c. = subcutaneous immunization with OVA and CFA; ear challenge = antigen challenge into ear pinnae; (N) = number of mice in group. Ear swelling values $\pm$ SEM of each group of mice are presented on the ordinate. *Significant difference ($P \leq 0.05$) between indicated groups. N.S. indicates no significant difference. Data presented in columns 4 to 9 are representative of three experiments.

eye interferes with immune-privileged mechanisms in both eyes.

RLB Allows for Immunization via the Eye

Antigen inoculation in the anterior chamber of the eye of an unmanipulated mouse does not induce an immune response but does induce tolerance to that antigen. However, it was not clear if the inoculation of antigen into the anterior chamber of the RLB mouse was a null event or actually immunized the mouse. Thus, groups of mice received RLB, or not, 1 day before inoculation of antigen and were directly tested for immunization by challenging their ear with the same antigen a week later. Ear thick-

Figure 3. DH assay to test the effect of RLB on the ability to induce ACAID in the contralateral eye. The contralateral eye received an anterior chamber injection of OVA 1 day after laser burn; 7 days later, mice were immunized subcutaneously with OVA in CFA. Ear thickness values $\pm$ SEM of each group of five mice are presented. The treatment of the various experimental groups is indicated along the abscissa under each bar. N.S. indicates no significant difference. Significance at $*P \leq 0.05$. Data are a representative experiment from three similar experiments.

Figure 4. DH assay to test if anterior chamber inoculation immunizes after RLB. **A:** Antigen inoculation (subcutaneously) of RLB eye immunizes. First bar is negative control; second bar is positive control for DH; third bar is ACAID; fourth bar shows that when antigen is inoculated into the anterior chamber after RLB it is immunizing. The thickness of the ear is shown on the ordinate. The treatment of the experimental groups is indicated under the abscissa below each bar. Significant difference ($*P \leq 0.05$) between two groups. N.S. indicates no significant difference. The experiment was performed three times. **B:** Antigen inoculation (anterior chamber) in untreated contralateral eye of RLB mice does not immunize. Mice received RLB to the right eye. One day later, OVA was inoculated into untreated mice (third bar) or (fourth bar) the left eye of RLB mice. One week later, ears were challenged with OVA and ear thickness was a measure of DTH. Negative control, first bar; positive control, second bar; N.S. indicates no significant difference ($*P \leq 0.05$).

ness was measured 24 hours later and compared with the thickness of the ear before being challenged (Figure 4A). We observed that the mice that received RLB before the anterior chamber inoculation of antigen showed increased ear thickness when challenged locally with the antigen. Thus, the RLB not only interfered with the mechanisms that induce tolerance in the eye but also allowed the ocular route to induce an immune response.

Inoculation of Antigen into the Nonburned Eye

Since we observed that antigen inoculation into the anterior chamber of the RLB-treated eye was immunizing,

we wondered if the inoculation of antigen into the anterior chamber of the contralateral eye of RLB mice was also immunizing. To test this, groups of mice received RLB, or not, to the right eye. One day later, OVA were inoculated in the contralateral (left) eye. As before, 1 week later mice were challenged with antigen in the ear pinnae. We observed that the mice that received RLB before OVA inoculation into the contralateral eye did not show an increase of ear thickness (Figure 4B). Thus, RLB-induced mechanisms abrogate the ability to induce immune deviation (ACAID) via the anterior chamber and may be different in the burned and nonburned eye.

RLB Changes the Functional Phenotype of the Indigenous APC

It is thought that antigen that is inoculated into the anterior chamber is carried to the spleen by the indigenous F4/80⁺ APCs.¹⁷ Previously, we reported that the F4/80 protein expressed by the APCs was required for the induction of ACAID and had a correlation with the APC being tolerogenic. F4/80⁺ APCs are required for both ACAID and low-dose oral tolerance.¹⁸ Furthermore, the F4/80 marker is never found in areas of lymphoid tissue that are devoted to immune response induction. These facts suggested to us that if the anterior chamber route of antigen delivery becomes immunizing, the indigenous APCs might lose their F4/80 marker after RLB burn. Because it is difficult to monitor the cells within the eye, we collected peripheral blood cells or spleen cells to evaluate changes in F4/80 expression after anterior chamber inoculation in RLB-treated and nontreated mice. Knowing that F4/80⁺ cells increased in both the peripheral blood and the spleens of anterior chamber inoculated mice,¹⁹ we determined if the F4/80⁺ APC population increased in RLB mice after anterior chamber inoculation of antigen (Figure 5, A and B). We observed that unlike the naive mice, RLB mice showed no increase in F4/80⁺ cells 3 days after anterior chamber inoculation of antigen. The CD11b⁺ + APCs were less affected by the RLB but did not increase as much as they did in the anterior chamber-inoculated mice. Because it is known that F4/80⁺ cells from peripheral blood or spleen of ACAID mice transfer ACAID,^{17,19} other studies were done to show that splenic adherent cells harvested from untreated (but not mice that received RLB) mice that were inoculated with OVA in the anterior chamber were able to transfer tolerance (Figure 5C).

AqH Is No Longer Immunosuppressive after RLB

AqH is known to be immunosuppressive, to contain TGF- β , and to contribute to the maintenance of the immune-privileged environment of the eye.^{20–22} Because RLB allows for inflammation within the eye, we postulated that RLB induced major changes in the composition of AqH. Wilbanks and colleagues²⁶ showed that AqH treatment of APC, in the presence of antigen *in vitro*, altered

Figure 5. DH assay to test if APC from anterior chamber inoculated mice transfer tolerance. **A:** Flow cytometry analysis of F4/80⁺ and CD11b⁺ cells in peripheral blood mononuclear cells after anterior chamber inoculation of nontreated or RLB mice. Peripheral blood mononuclear cells were collected (3 days after anterior chamber) from mice that were previously splenectomized so that cells remained in circulation. Splenectomized mice received RLB or not. Percentage of positive cells is shown in each panel. **B:** Ratio of cells with markers in peripheral blood mononuclear cells from RLB and untreated mice. Ratio of markers is shown above each panel. Treatment of the mice from which the cells were harvested is indicated under the abscissa below each bar. **C:** Peripheral APCs from mice that received RLB do not transfer tolerance. APCs were transferred intravenously from mice that received RLB before ACAID induction by anterior chamber injection of antigen. Six days later, spleens were removed and cells dissociated and incubated on plastic for 90 minutes. Adherent cells were collected and transferred intravenously to naive mice. Recipient mice were immunized a week before antigen challenge into the ear pinnae. Ear thickness (ordinate) was measured as an indication of DH. Significance; N.S. indicates no significant difference. (* $P \leq 0.05$).

their antigen-presenting ability in such a way they acquired the ability to induce tolerance instead of immunity to that antigen.²³⁻²⁶ To test the immunoregulating function of AqH, the fluid was collected from 15 eyes of nontreated mice, 15 RLB treated eyes, and 15 contralateral eyes, then separately co-cultured with OVA-pulsed PECs. Unlike AqH from eyes of naive mice, *in vitro* analyses of the AqH samples harvested 24 hours after RLB

Figure 6. Capacity of AqH to induce tolerogenic PECs. Thioglycolate-induced PECs were cultured for 18 hours with AqH collected from experimental and control animals. **A:** Flow cytometry analyses of APC activation marker, CD40. Treatment of the PECs is given within each panel. **Top:** Expression of CD40 in the PECs under various control conditions. The last panel in top row shows that TGF- β is capable of down-regulating CD40, *in vitro*. **Bottom:** The effect of various samples of AqH collected from wild-type (WT) and RLB mice on CD40 expression. Percentage of cells that are positive for CD40 is indicated above the population marker in each block. **B:** RT-PCR analyses of IL-10 mRNA expression. PECs cultured with AqH were examined by RT-PCR. Treatment of the cells before mRNA analysis is indicated under the abscissa. *In vitro* imaging of gel is lined up with the densitometry reading (ordinate) of IL-10: β -actin ratio.

from either the RLB or contralateral eye were unable to modulate the antigen-presenting ability of OVA-pulsed PECs as assessed by their expression of CD40, a critical co-receptor for immune activation (Figure 6A).

Because APC-derived IL-10 is essential for ACAID induction,²⁷ other experiments tested if AqH from RLB mice was able to induce the production of IL-10 in the co-cultured APCs (Figure 6B). OVA pulsed PECs were cultured (24 hours) with AqH from naive, RLB-treated, or RLB contralateral eyes and assessed for IL-10 mRNA by RT-PCR. We observed that IL-10 mRNA expression was increased in APCs that were cultured with AqH from nontreated eyes but not increased in APCs cultured with AqH collected from either eye of mice that received RLB to only one eye. These data support the postulate that RLB alters the characteristics of AqH so that it is no longer able to induce tolerogenic changes in the APCs.

Discussion

Although the eye is an immune-privileged site and has mechanisms to interfere with the development of immune inflammation, under certain pathological conditions, ocular immune privilege is terminated and vigorous inflammation occurs.^{8,28} This process contributes to the pathogenesis of many eye diseases. Understanding mechanisms that abrogate immune privilege may lead to novel therapies to restore immune regulatory mechanisms in the eye and other sites of autoimmunity.²⁹

Here we report that mechanisms that contribute to immune privilege are disrupted in the eye when the retinal pigment epithelial cells are damaged by laser burn. Surprisingly, damage to the retina in one eye had altering effects on immune privilege in the nontreated eye. There is general agreement that immune privilege is a constitutive feature of the anterior chamber in normal eyes, and there is a general expectation that when inflammation occurs in the anterior segment of the eye immune privilege will be lost.³⁰ Although we were surprised that laser damage to the back of the eye affected immune privilege of mechanisms in the front of the eye, studies using experimental uveitis models of ocular inflammation had shown that posterior inflammation disturbed the anterior chamber and robbed it of its capacity to support ACAID induction.^{31,32} However in all these cases, unlike our RLB model, the loss of ACAID was transient.³³ Moreover the conclusion from the effects of uveitis on immune privilege was that immune privilege is surprisingly resistant to abolition by intraocular inflammation.³³ Additionally, unlike uveitis models that were explored previously, in the RLB models only one eye is inflamed yet immune privilege is breached in both. Therefore, a new immune-privileged mechanism maybe revealed by studying this model.

Immune privilege is mediated by both active and passive mechanisms.⁸ The eye is privileged in part because of the blood ocular barriers that include the iris, ciliary body, and retinal pigment epithelium as well as the retinal microvasculature. Local immunosuppression is provided by immunomodulatory and anti-inflammatory factors in ocular fluids,³⁴ and on parenchymal cell surfaces.^{35,36} Constitutive expression of FasL on intraocular cells provides protection by inducing apoptosis of activated lymphocytes and neutrophils that transgress the borders and might damage ocular tissues.³⁷ Systemic factors also contribute to immune privilege in that eye-derived APCs carry antigens to the spleen where they induce antigen-specific T-regulatory cells that affect both local and peripheral tolerance to the eye-derived antigens.

Thus, with the overlapping mechanisms that contribute to immune privilege, inoculation into the anterior chamber is a route that consistently leads to tolerance. Thus, when we observed that RLB interfered with development of tolerance in the ACAID model we thought is was a null event. However, we noted the inoculation of antigen into the anterior chamber of the burned eye led to immunization and a DH response. This is remarkable in that we did not add adjuvant, a substance that is absolutely necessary even if immunization is through the skin.^{38,39} This may suggest that the RLB leads to release of local mol-

ecules (maybe stress proteins) that act as adjuvants. This notion raises new questions about regulation of immune privilege.

To explain the loss of ACAID in the burned eye after RLB, we postulated that the quality of the AqH was altered by the break in the barrier and subsequent infiltration of inflammatory cells caused by the burn. It is known that soluble factors within the AqH, such as macrophage-derived migration inhibitory factor (MIF),³⁴ TGF- β ,²⁰ and neuropeptides,^{22,40} all contribute to the immune privilege of the eye⁸ through their immunosuppressive and anti-inflammatory effects.

Because our focus was to extend previous studies and understanding of immune privilege, we chose to examine the functional affects of AqH on APCs. Similar to other factors found in AqH, TGF- β is produced locally within the eye, and is thought to be the most important agent responsible for modulating APC toward tolerance induction.^{23,41–43} Here, we observed that AqH from eyes of nontreated mice converted APCs into tolerogenic APCs unlike the AqH from either eye of mice that had received RLB in one eye. Furthermore APCs collected from the spleen after anterior chamber inoculation of antigen in mice receiving RLB were unable to transfer tolerance. These observations suggest that RLB induces changes in AqH that affect the tolerogenic function of the indigenous APC in the eye.

It is easier to explain the abrogation of ACAID phenomenon in the burned eye than in the contralateral eye, because inflammatory cells enter the eye through the break in the blood ocular barrier caused by the burn. Inflammatory cells by definition release inflammatory cytokines that then alter the composition of the AqH⁴⁴ and its capacity to induce tolerance. But, because the barrier is apparently intact in the contralateral eye, we pose that the mechanisms that caused the change in tolerogenic capacity of the AqH collected from the non-burned eye are different and unrelated to the presence of inflammatory cytokines produced by recruited cells in the burned eye.

Neuronal signals from the laser-damaged RPE cells of the treated eye may have a modifying influence on the contralateral eye. It is known that RPE cells produce neural messages^{45,46} that are able to travel between eyes via the nerves. Thus, the possibility is raised that laser burn damage to the RPE initiates a process that spreads to nearby undamaged RPE cells as well as to the RPE in the contralateral eye. Therefore, tolerogenic neural messages could be lost between the eyes and lead to the loss of an ability to develop ACAID in the contralateral eye. Such an idea raises concerns about the safety of laser treatment even if it is targeted to selected RPE cells.^{47,48}

Because RPE cells also make immunosuppressive cytokines another idea that is unveiled by this model is that the RLB damage to the RPE may interfere with nonneuronal immunosuppressive factors⁴⁹ and the posterior eye (RPE) may be a major source of the immunosuppressive factors found throughout the AqH in both eyes.⁵⁰ We

suspect that if the mechanisms that led to the RLB-associated abrogation of immune privilege were understood it might be possible to restore immune privilege to the inflamed eye. Even though RLB is commonly used as treatment in inflammatory disease of the eye, few studies have been done to determine whether the immune privilege is altered by such therapeutic measures. This may be a mute point, however, because an eye that warrants laser burn treatment may already have its immune-privileged mechanisms compromised. Then the question becomes, "Is the additional interference of immune privilege by laser burn helping or hindering the restoration of immune stability in the eye?"

Understanding how to restore immune regulation in the face of immune inflammation might lead to novel therapeutic strategies for immediate intervention in patients with accidental laser burn or trauma to an eye and may prevent the appearance of sympathetic ophthalmia. Sympathetic ophthalmia is a condition in which trauma to one eye leads to immune inflammation and potentially blindness in the nontraumatized eye^{51,52} because of a loss of its immune privilege. At the very least this puzzling situation might benefit from future studies exploiting RLB model as a model for sympathetic ophthalmia.

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