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Ocular autoimmunity: the price of privilege?

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Summary: The eye is the prototypic immune-privileged organ. Its antigens were once believed to be expressed exclusively in the eye, which resides behind an efficient blood–organ barrier, and were believed to be unknown to the immune system. Self-tolerance to ocular components was therefore believed to be based not on immune tolerance but on immune ignorance. It is now known that the relationship between the immune system and the eye is much more complex. On the one hand, immune privilege is now known to involve not only sequestration but also active mechanisms that (i) inhibit innate and adaptive immune processes within the eye and (ii) shape the response that develops systemically to antigens released from the eye. On the other hand, retinal antigens are found in the thymus and have been shown to shape the eye-specific T-cell repertoire. However, thymic elimination of self-reactive T cells is incomplete, and such 'escapee' T cells are tolerized in the periphery as they recirculate through the body by encounter with self-antigen in healthy tissues. Due to the relative inaccessibility of the healthy eye to the immune system, peripheral tolerance mechanisms may not operate efficiently for ocular antigens, leaving a weak link in the homeostasis of tolerance. The case shall be made that although immune privilege protects vision by keeping the immune system at bay, a potential for developing destructive anti-retinal autoimmunity may be the price for the day-to-day protection afforded by immune privilege against inflammatory insults.

Keywords: *experimental autoimmune uveitis, uveitis, immune privilege, immunological tolerance, autoimmune disease*

Introduction

It has been known for a long time that the eye has a special relationship with the immune system, known as immune privilege. An overview of and a historical perspective on the concept of privilege has been presented earlier in this volume (1), so the present review deals with the privilege phenomenon specifically as it pertains to the subject of ocular autoimmunity. The immune privilege of the eye is a complex phenomenon, involving many layers and mechanisms: (i) physical barriers prevent entry and exit of larger molecules such as proteins from the eye; (ii) cell-bound and soluble immunosuppressive factors within the eye inhibit the activity of immune-competent cells that may gain entry; and (iii) protein antigens released from

a damaged eye elicit deviant systemic immunity that limits the generation of proinflammatory effector cells [reviewed by Streilein (2)]. Acting in concert, these elements serve to create a milieu designed to protect the delicate visual axis from damage by inflammatory processes that in any other organ would not carry adverse functional consequences. Based on accumulated evidence from rodent studies, it is widely accepted that breakdown of immune privilege contributes to bystander damage from infection, to rejection of corneal grafts, and to development of uveitis [reviewed by Streilein (2) and Niederkorn(3)]. However, the ease with which it is possible to elicit autoimmunity in experimental animals to antigens originating from the retina puts in question the role of immune privilege as an effective barrier against ocular autoimmunity.

A premise that must be examined in this context is the extent and nature of self-tolerance to retinal antigens. T cells that are able to proliferate to retinal antigens are easily detectable in peripheral blood of healthy humans. de Smet *et al.* (4) found a frequency of up to 4 per 10 million peripheral blood lymphocytes able to proliferate to retinal arrestin [retinal soluble antigen (S-antigen)] by limiting dilution. This number is probably a gross underestimation, as only some autoreactive cells proliferate, and methods that do not rely on proliferation detect 20-fold higher antigen-specific T-cell frequencies (5). Furthermore, this frequency in the naive repertoire represents only one retinal antigen out of more than 10 shown to be uveitogenic in animals (6), suggesting that tolerance to ocular antigens is deficient.

Although immune privilege and ocular autoimmune disease have been known and studied since the early part of the century, recent advances in research tools available to study basic questions in immunology are making it possible to examine these issues directly. Thanks to state-of-the-art approaches using genetically engineered mice, combined with sophisticated cell labeling and tracking methodologies, great strides have been made in the last decade in the understanding of both immune privilege and autoimmunity in the eye. This review critically examines the current thinking on the relationship between the eye and the immune system and how immune privilege might affect tolerance and immunity to endogenous ocular antigens in health and in disease. The important role of immune privilege in corneal transplantation (7) is not discussed in this review, as it is covered by others elsewhere in this volume (1, 8).

Elements and mechanisms of immune privilege

Immune privilege, a term coined by Medawar (9) in the mid-1900s to describe the acceptance of foreign tissue grafts placed in the anterior chamber of the eye, is now known to be a highly complex phenomenon. Medawar favored the view that immune privilege is due primarily to the separation of the eye from the immune system (9). In recent decades, however, immune privilege has been extensively explored by many investigators, the most prominent of whom was J. Wayne Streilein (recently deceased), who have demonstrated unequivocally that separation is only a part of the complex phenomenon of immune privilege. Since immune privilege most assuredly did not evolve to prevent graft rejection, it has been proposed that its primary purpose is to control immune and inflammatory processes, such as those caused by responses to infectious agents, that have the potential to cause severe bystander damage to ocular tissues and compromise vision (2). The model of privilege that emerges from many studies is a three-tiered system composed of the following elements (*Table 1*): separation, inhibition, and regulation.

Separation

Sequestration can be considered a 'passive' mechanism of privilege. The posterior chamber of the eye becomes separated from the immune system early in ontogeny by an efficient blood-retinal barrier composed of the retinal pigment epithelium (RPE) and the retinal vascular endothelial cells. Vascular endothelial barriers also limit diffusion of molecules from the blood into the anterior chamber. The blood-ocular barrier is very selective and excludes molecules even as small as 376 Da, which is the size of sodium fluorescein used routinely in the clinic to assess the integrity of the blood-retinal barrier (fluorescein angiography, http://www.mrcophth.com/ffainterpretation/ffaprinciples.html). Furthermore, although the external surface of the eye and the subconjunctival space is drained to the regional (preauricular) lymph nodes, the interior

Table 1. **Elements and mechanisms of immune privilege**

Effect	Site	Nature	Mechanisms	References
Separation	Local	Passive	Blood-retinal barrier, lack of efferent lymphatics	9, 11
Inhibition	Local	Active	Soluble and cell-bound immunoinhibitory substances within the eye, paucity of MHC class-II-expressing APCs	13, 18, 19, 21, 23, 24, 26, 28
Regulation	Systemic	Active	ACAID and ACAID-like regulatory phenomena	32, 37

of the uninflamed eye is believed to have no direct lymphatic drainage. The issue of lymphatic drainage has been questioned recently based on the data showing that in mice systemically infused with OT-II [ovalbumin (OVA) specific] T-cell receptor (TCR) transgenic (Tg) T cells, injection of the OVA peptide into the posterior segment was followed by a specific response in the submandibular lymph nodes (10). However, some external leakage of the peptide antigen may well have occurred through the point of injection, so it is difficult to extrapolate to the intact eye from these experiments. Significantly, Dullforce *et al.* (11) have reported that antigen-presenting cells (APCs) from the anterior chamber do not migrate to the regional lymph node.

Inhibition

Inhibition is manifested as dampening or prevention of immune and inflammatory responses within the eye. Although the blood-retinal barrier effectively excludes protein molecules, including antibodies and complement components, it is not effective against activated lymphocytes (12). However, an infiltrating lymphocyte that enters the eye encounters a profoundly hostile environment, which is not conducive to supporting their activation and function. First, the posterior part of the eye has a paucity of major histocompatibility complex (MHC) class-II-positive cells that might act as APCs. Second, ocular resident cells both in the posterior segment and in the anterior segment, which comprise anatomical barriers that an infiltrating lymphocyte must cross, or that form structures with which it is likely to come in contact, are able to inhibit activated lymphocytes by contact-mediated mechanisms. These mechanisms include retinal glial Müller cells, RPE cells, corneal endothelial cells, and iris/ciliary body epithelial cells (13–17). Some of the inhibitory cell-surface-associated molecules have been identified, including membrane-bound transforming growth factor-β (TGF-β), Fas ligand (FasL), B7–CTLA4 interaction, galectin-1, thrombospondin, and some remain to be identified (13, 17–22). The role of Fas/FasL interactions in immune privilege is discussed elsewhere in this volume (8). Third, the ocular fluids contain a number of immunoinhibitory molecules, beginning with microgram quantities of TGF-β, mainly TGF-β2, produced by resident ocular cells (23). Several immunosuppressive neuropeptides, including α-melanocyte-stimulating hormone (α-MSH), calcitonin-gene-related peptide, vasoactive intestinal peptide, and somatostatin, have been demonstrated in aqueous humor, as has been migration inhibitory factor (MIF) (23, 24). Finally, an interleukin-10 (IL-10)-like molecule has also been identified at the messenger RNA (mRNA) level in eyes of resistant but not susceptible rat strains, and it may constitute a little explored aspect of immune privilege (25). These soluble factors inhibit the activation and function not only of lymphocytes but also of elements comprising the innate immune system, including natural killer cells, macrophages, and granulocytes (26). Much about the precise nature of these interactions, both soluble and membrane bound, and the cellular processes they affect still remain to be explored, but their purpose is one, which is to protect the eye from direct and indirect damage inflicted by activated leukocytes and their products.

Last but not least, inhibitors of complement activation are constitutively expressed within the eye and are functionally active (27, 28). Their importance in maintaining the immunosuppressive environment of the eye is demonstrated by the finding that inflammation is significantly enhanced in their absence (29, 30). However, the low level of complement activation present in the eye, which is tightly regulated by the complement regulatory proteins, might actually contribute to the immunosuppressive environment. In an eye-derived tolerance model, ligation of the complement activation product iC3b to its receptor on APCs results in production of TGF-β2 and IL-10 from these cells. This alteration in APC phenotype was found to be essential for systemic induction of eye-derived tolerance and conceivably could also inhibit local antigen presentation in the eye (31).

Regulation

This last point concerning a role for activated complement products in systemically expressed tolerance to antigens coming from the eye brings us to the phenomenon known as anterior-chamber-associated immune deviation (ACAID). ACAID is the first discovered and the best studied (though certainly not the only) model of eye-derived tolerance. It can be considered as the active, systemic component of immune privilege. A foreign protein injected into the anterior chamber of the eye is not ignored by the immune system; instead, it elicits a deviant immune response characterized by a dampened delayed-type hypersensitivity (DTH) response, elicitation of non-complement-binding antibodies, and production of antigen-specific regulatory T cells (Tregs) (32). The immune network leading to the elicitation of ACAID involves exit of antigen-bearing $F4/80^+$ APCs from the anterior chamber and their obligate migration to the spleen. There they recruit natural killer T cells through a process requiring macrophage inflammatory protein 2 and depending on CD1d. Marginal zone B cells as well are a required part of this multicellular complex, which culminates in the induction of $CD4^+$ and $CD8^+$ Tregs. The former inhibit acquisition of immunity (afferent acting) and the latter suppress expression of immunity (efferent acting). The posterior

segment of the eye also appears to enjoy an immune-privileged status, and antigens placed in the subretinal space elicit a deviant immune response, although its privilege may be less extensive than that of ACAID (33–36). The privilege in the posterior segment is less well studied than ACAID, and it is unclear whether it shares all of the same mechanisms.

A valid criticism against the ACAID phenomenon as broadly representative of an eye-derived tolerance is that it is not a physiological situation; the vast majority of information on mechanisms has been obtained by studying the effects of OVA injected into the anterior chamber. However, eye-derived tolerance involving Tregs to retinal antigen can be demonstrated in mice that have recovered from experimental autoimmune uveitis (EAU) (37). Induction of these Tregs was dependent on the presence of the eyes throughout the disease process, but the nature of the postrecovery Tregs appeared to be distinct from those induced by ACAID (37).

Autoimmunity in the eye: human and experimental autoimmune uveitis

Despite and perhaps in part because of its immune-privileged status, the eye is subject to autoimmune attack. Autoimmune uveitis in humans comprises a group of potentially blinding ocular inflammatory diseases affecting more than 150 000 persons annually in the United States (38, 39). Either the anterior or the posterior compartment of the eye can be preferentially affected. Anterior uveitis is mainly localized in front of the lens. It is much less destructive to vision than posterior uveitis, and most forms are easily treated. Consequently, it has been less well studied immunologically than posterior uveitis, and the putative autoantigens driving it are in question. Posterior uveitis (uveoretinitis) is the form more likely to result in loss of vision, due to irreversible damage to the neural retina and adjacent structures. Some types of posterior uveitis involve only the eye, whereas in others, uveitis is part of a systemic syndrome. Sympathetic ophthalmia and birdshot retinochoroidopathy are examples of the former, whereas Behçet's disease, sarcoidosis, and Vogt-Koyanagi Harada disease are examples of the latter. Uveitis patients frequently exhibit lymphocyte responses to retinal antigens. Responses most often are seen to retinal arrestin (S-antigen), but responses to other components of the retina have also been described. Although it is unclear whether these responses are the primary cause of uveitis or its result, they are believed to fuel the progression of the disease. The etiologic causes that trigger uveitis are unknown except in the case of sympathetic ophthalmia, which is precipitated by ocular trauma followed by a destructive inflammation in the non-traumatized 'sympathizing' eye. It is believed that antigens released from the traumatized eye find their way into the draining lymph node and elicit systemic immunity. In uveitic disease that cannot be linked to a trauma, it is believed that lymphocytes capable of recognizing retinal antigens are primed in the periphery by a cross-reactive microbial stimulus.

A role for retinal antigens in the etiology and progression of uveitic disease is supported by strong human leukocyte antigen (HLA) associations in many types of uveitis, by up to a 50-fold rise in the frequency of S-antigen-specific T cells in the blood of uveitis patients, and by the finding that T-cell targeting strategies and antigen-specific oral tolerance therapy with S-antigen are able to modulate disease progression (4, 40). The role of retinal antigens in uveitis is also strongly supported by animal models. Immunization with retinal antigens incorporated into complete Freund's adjuvant elicits EAU in rodents and in non-human primates, and its histological appearance closely resembles uveitis in humans (41) (*Fig. 1*). There are a surprisingly large number of retinal antigens that have been shown to induce EAU, and new uveitogenic proteins continue to be reported. Proteins from the photoreceptor cell layer include the already mentioned S-antigen as well as interphotoreceptor retinoid-binding protein (IRBP), phosducin, recoverin, rhodopsin, and its illuminated form opsin. Uveitis can also be induced with proteins derived from the RPE and ocular melanin components. In mice, the antigen of choice to elicit EAU is IRBP, whereas in Lewis rats both IRBP and S-antigen elicit severe disease (42). A detailed review of different uveitis models and the antigens used to elicit them has recently been published (6).

Although there are species differences in susceptibility to particular antigens, the manifestations of disease and the cellular mechanisms appear similar across antigens and across species. EAU is a T-cell-dependent disease model that appears to be associated with a genetic predisposition to mount a T-helper 1 (Th1)-dominated adaptive response. Polarized uveitogenic Th1 cells [interferon-γ (IFN-γ) producing] are formidable effectors: significant EAU can be induced by as few as 500 000 cells from a Lewis rat T-cell line specific to an immunodominant epitope of S-antigen or a B10.RIII mouse T-cell line specific to an immunodominant epitope of IRBP (R. R. Caspi and P. B. Silver, unpublished data). The extent of involvement in EAU of the recently described IL-17-producing effector T cell (Th17) is still unclear and is currently being investigated. An indication that the Th17 effector may play a role in pathogensis is the ability of treatment with anti-IL-17 antibodies to ameliorate disease, and conversely, the enhanced IL-17 response to IRBP observed in the highly EAU-susceptible IFN-γ knockout (KO) mice (D. Luger, D. Cua and R. R. Caspi, unpublished results). IFN-γ

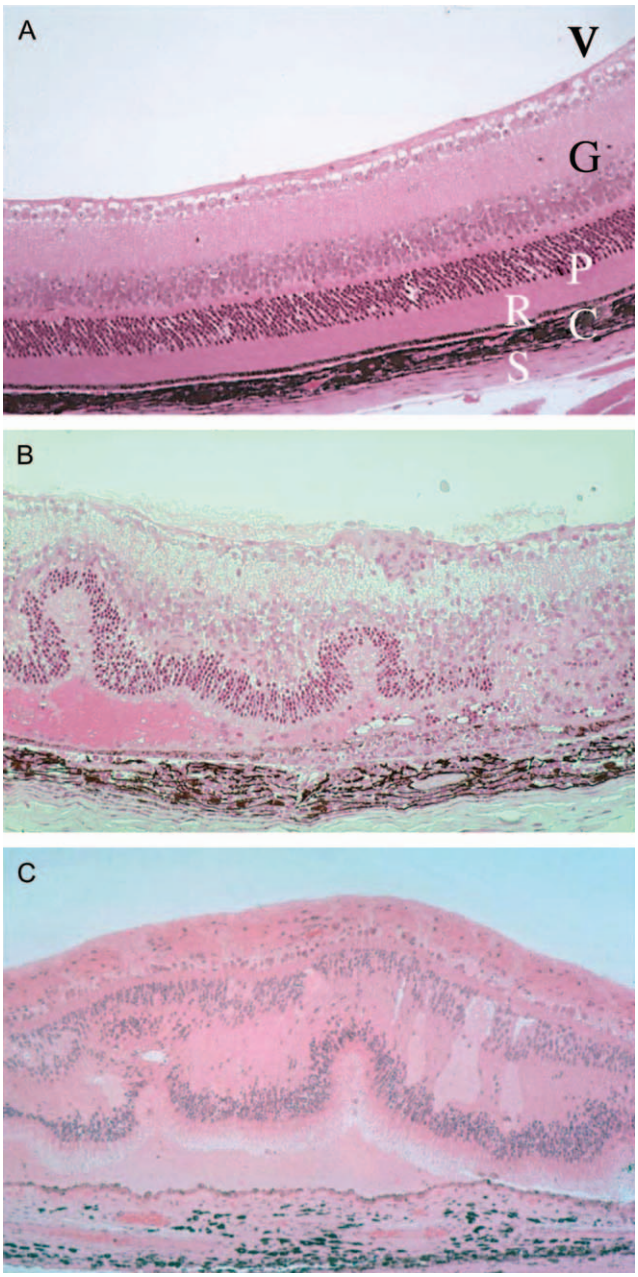

***Fig. 1.* Histological features of EAU in the mouse versus uveitis in human.** The photomicrographs show sections through the posterior pole of the eye. (A) Healthy mouse retina. V: vitreous; G: ganglion cell layer; P: photoreceptor cell layer; R: retinal pigment epithelium; C: choroid; S: sclera. (B) EAU induced in the B10.RIII mouse by immunization with IRBP in complete Freund's adjuvant. The retinal layers are disorganized. Lesions include loss of nuclei in the ganglion cell layer and the photoreceptor cell layer, prominent retinal folds, subretinal exudate with subretinal hemorrhage, vasculitis, damage to the retinal pigment epithelium, and inflammation of the choroid. (C) Uveitis in human (ocular sarcoidosis). Note similarity of lesions in (B) and (C). Photographs provided by Dr Chi-Chao Chan, Laboratory of Immunology, NEI. [This Figure was previously published in Drug Discovery Today: Animal Models (6). Copyright does not apply.]

KO mice are highly susceptible to EAU but lack a normal Th1 response; EAU in these mice was hitherto believed to be driven by a deviant, Th2-like effector response (43).

Irrespective of the precise effector type of the T-cell response, its very presence, the plethora of retinal antigens that elicit it, and the ease with which it is induced in susceptible animals upon appropriate immunization raise two central questions: (i) what is the nature and extent of tolerance to the autoantigens residing in the retina, and (ii) if immune privilege is such an effective barrier against induction and expression of immunity in the eye, why does it not prevent uveitis?

Self-tolerance to retinal antigens

Central tolerance

Self-tolerance to autoantigens is induced and maintained by a combination of central (thymic) and peripheral mechanisms. Antigens for most tissue-specific antigens are ectopically expressed in the thymus and determine the selection of the antigen-specific T-cell repertoire (44). The presence of retinal antigens in the thymus was first demonstrated by Egwuagu *et al.* (45), who presented evidence that the level of expression of S-antigen and IRBP in the thymus of a series of rat and mouse strains is inversely correlated with their susceptibility to EAU induced with that antigen. Thus, EAU-resistant mice expressed higher levels of IRBP in the thymus than mice that developed EAU when immunized with IRBP. In the most susceptible strain, B10.RIII, expression could not be detected by conventional polymerase chain reaction (PCR). In contrast, all tested mouse strains expressed relatively high levels of S-antigen in their thymi, in line with the observation that mice as a species appear to be resistant to EAU induced with S-antigen. We subsequently demonstrated that even in the most susceptible B10.RIII mouse strain, IRBP can be detected in the thymus at the single-cell level (46). By using IRBP-deficient mice and thymic transplantation, it was possible to demonstrate directly that this low level of IRBP expression has a functional significance, in that it eliminates pathogenic T-cell specificities and reduces the threshold of susceptibility to EAU. Thus, wildtype B10.RIII mice grafted with an IRBP KO thymus not only had a demonstrably expanded T-cell repertoire but also developed high EAU scores when immunized with a dose of IRBP that induced only minimal or no disease in wildtype mice with a wildtype thymus (46). Whether the T cells specific for epitopes to which responsiveness is eliminated by wildtype mice are physically deleted or whether some are anergized (and perhaps become regulatory cells, see ahead) remains to be determined, as mice with Tg TCRs for native retinal antigens are not yet available. However,

it is possible to directly demonstrate thymic deletion of 'retina-specific' T cells in animals expressing hen egg lysozyme as a neoantigen under the control of a retina-specific promoter such as IRBP or rhodopsin (47, J. V. Forrester, personal communication).

Expression of the retinal S-antigen in the thymus localizes to thymic medullary and thymic cortical epithelial cells (mTECs and cTECs, respectively) (48). It was not identified in thymic dendritic cells but was one of the few antigens detected in thymic macrophage-like cells. The significance of this localization is not clear. In contrast, IRBP is found only in mTECs (49). Expression of uveitis-relevant antigen(s) in the thymus is under control of the AIRE (autoimmune regulator) protein, a transcription factor that controls the ectopic expression of many, but not all, tissue antigens in TECs (50). Mice deficient in the AIRE protein develop anti-retinal antibodies that localize to the photoreceptor cell layer of the retina. They also exhibit cellular infiltrates and photoreceptor cell damage in the eye, which appear histologically indistinguishable from EAU (50). Recent data indicate that the specificity of this spontaneous response appears to be directed against IRBP (J. DeVoss, R. R. Caspi, and M. S. Anderson, manuscript submitted). Interestingly, this finding is reminiscent of another spontaneous EAU model, which occurs in nude mice grafted with a neonatal rat thymus. These mice develop an autoimmune disease syndrome that involves multiple organs, among them the eye, and exhibit an immune response directed against photoreceptors with specificity to IRBP but not to S-antigen (51).

The thymus as the purveyor of central tolerance not only negatively selects the effector repertoire but also generates 'natural' Tregs (nTregs) that protect from tissue-specific autoimmunity (52). In view of the ability of the eye to generate its own specialized regulatory circuits, one might wonder whether the function of such cells to control ocular autoimmunity might not be totally redundant. However, depletion of $CD4^+CD25^+$ T cells from naive B10.RIII mice that are subsequently challenged for EAU considerably lowers their threshold of susceptibility to the disease (46, 53). In addition, these cells may be implicated in the resistance of some low-susceptibility strains to EAU. For example, depletion of $CD4^+CD25^+$cells from the moderately susceptible C57BL/6 mice or from the resistant BALB/c mice permits development of high disease scores in both strains after IRBP challenge (R. S. Grajewski and R. R. Caspi, manuscript in preparation). The thymic origin of nTregs that protect from EAU remains to be directly demonstrated but is supported by the finding that their functional reconstitution after depletion is delayed in adult thymectomized mice (46). The generation of IRBP-specific nTregs requires endogenous expression of IRBP, as they are absent in IRBP KO mice. Interestingly, however, EAU can be regulated also by nTregs of unrelated specificities, which may be activated through innate immunity receptors and act in a bystander fashion (53).

While the findings discussed above show that central tolerance has a major role in blunting the responses to ocular autoantigens, in uveitis-susceptible individuals, its effectiveness is obviously insufficient. Thymic negative selection is never 100% effective, and many autoantigen-specific T cells escape from the thymus into the periphery (54). The reasons for this escape can be many, including the affinity of the TCR–antigen interaction and the amount of the uveitogenic autoantigen expressed in the thymus. In a series of mouse and rat strains with differing susceptibility to EAU, susceptibility roughly correlated with the level of thymic expression of the retinal antigen (45). Differences in level of expression of retinal antigens in human thymi as well have been observed and are suggested to contribute to a predisposition to development of ocular autoimmune disease (55). A recent study also pointed out that central tolerance induction is less effective in neonates than in older animals, suggesting an increased opportunity during early life for export of autoreactive cells from the thymus (54).

Peripheral tolerance

Self-reactive T cells that escaped deletion in the thymus normally get a second chance at tolerance peripherally, as they recirculate through the tissues. Upon encounter of their cognate antigen in healthy tissues, in the absence of costimulatory and 'danger' signals, such autoreactive T cells are turned off, so that they become less able to be activated if they subsequently encounter the same antigens under inflammatory conditions.

It is uncertain whether any unique ocular antigens reside in the anterior part of the eye. The retina, on the other hand, is known to contain numerous tissue-restricted antigens, including S-antigen, IRBP, and others (41, 56). These are for the most part highly conserved proteins involved in the visual cycle that are not found elsewhere in the body (with the exception of the pineal gland or 'third eye'). Peripheral tolerance to retinal antigens appears to be weak. In the blood of healthy humans, proliferation-based precursor frequency analysis of S-antigen-specific T cells detects the rather high frequency (for an autoantigen) of 4 per 10^7 cells (4), which could be an underestimation of the actual frequency by a factor of about 20-fold (5). This conclusion is strongly supported by several studies showing that forced expression of retinal antigen in the periphery through a variety of gene transfer methodologies

results in profound resistance to induction of EAU. McPherson *et al.* (57) transduced Lewis rat bone marrow cells with retinal S-antigen, which is highly uveitogenic in this strain. Syngeneic recipients grafted with these bone marrow cells were resistant to EAU, and resistance correlated with the expression of S-antigen in their hematopoietic cells, detectable by PCR (57). Similarly, Agarwal *et al.* (58) expressed the major pathogenic epitope of IRBP in peripheral B cells of mice through retroviral technology. Again, recipients of these cells became refractory to EAU and remained so for at least 10 months (58 and unpublished data). Lastly, systemic injection into mice of a naked DNA plasmid encoding a large fragment of IRBP conferred resistance to induction of EAU with an epitope contained in the plasmid (P. B. Silver and R. R. Caspi, manuscript submitted).

Thus, peripheral tolerance may be the 'weak link' that is unable to compensate for deficits in central tolerance that allow export of retina-specific T lymphocytes into the periphery. These untolerized T cells remain available to be activated by (in)appropriate exposure to a retinal antigen or a cross-reactive mimic in the presence of an adjuvant effect provided by a concomitant infection or inflammation. Despite the presence of preexisting nTregs, if a sufficient number of effectors are activated, they will migrate and find their way into the eye. A minuscule number of such uveitogenic effector T cells, which appear to reach the eye at random, is sufficient to trigger the entire inflammatory cascade culminating in EAU (12). These checkpoints in the development and breakdown of self-tolerance to retinal antigens that lead to uveitis are depicted in *Fig.* 2 and represent information compiled over the years from studies in many laboratories.

How does immune privilege affect ocular autoimmunity?

A large body of experimental evidence supports the notion that immune privilege has an important role in directly and indirectly dampening inflammatory processes occurring in the eye (2, 18, 26, 32). The most frequent inflammatory insult that an organism would encounter would undoubtedly be

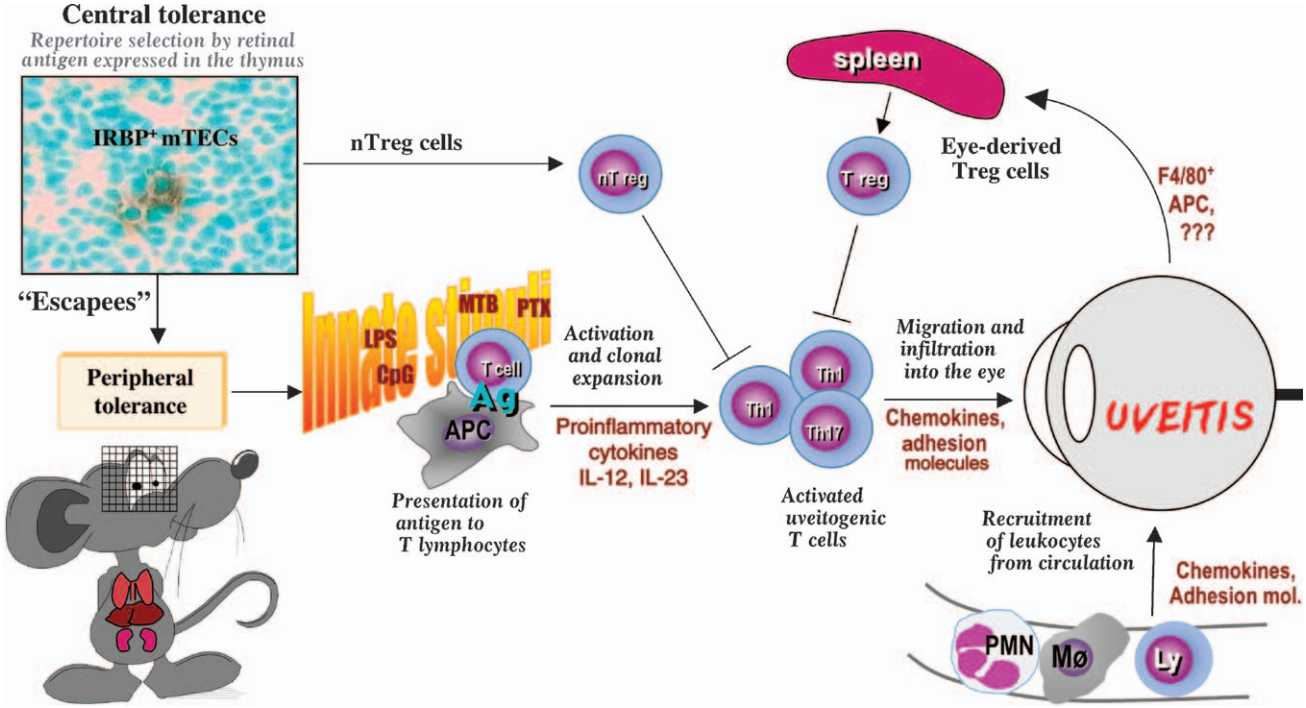

***Fig.* 2. Critical checkpoints in tolerance and autoimmunity to retinal antigens.** Shown is a schematic representation of the checkpoints and regulatory events in the process of ocular autoimmunity. Incomplete elimination of retina-specific effector precursor T cells in the thymus, combined with deficient peripheral tolerance, leads to a circulating pool of non-tolerized T cells that can be triggered by exposure to a retina-derived or cross-reactive antigen presented in the context of inflammatory danger signals. This leads to a differentiation of the activated T cells to an autoaggressive Th1 or Th17 phenotype. nTregs are exported from the thymus along with the effector precursors and inhibit their activation and clonal expansion in an antigen-specific and in a bystander fashion. Activated effector T cells migrate and extravasate at random, and some reach the eye. Recognition of the cognate antigen in the tissue is followed by downstream events culminating in a breakdown of the blood-retinal barrier, recruitment of inflammatory leukocytes, further amplification of the inflammatory process, and uveitis. Retina-derived antigens released from the damaged tissue induce generation of antigen-specific Tregs in a process requiring the spleen, which help establish a new type of balance and maintain functional tolerance.

caused by infectious microorganisms. Because good vision is one of the strongest selective pressures, the survival benefit of day-to-day protection of the delicate ocular structures overrides the disadvantage of increased vulnerability to less frequently encountered hazards, such as tumors that might arise within the eye. The special immunological status of the eye must affect the way that the endogenous antigens resident within the eye are seen by the immune system. In this section, we examine how the immune system 'sees' self-antigens found in the eye and the consequences of immunological privilege in terms of ocular autoimmunity.

Sequestration of retinal antigens limits acquisition of peripheral tolerance

The data discussed in the preceding section provide strong evidence that peripheral tolerance to retinal autoantigens is deficient. We propose that this deficiency is due to sequestration of resident retinal antigens from the immune system by efficient local physical and biological barriers. As discussed above and summarized in *Table 1*, these barriers prevent free trafficking of cells and molecules between the healthy eye and the rest of the body. Thus, recirculating peripheral lymphocytes have no opportunity to encounter retinal antigens under non-inflammatory conditions and become tolerized.

The concept of sequestration of ocular antigens has recently been challenged, mostly based on the ACAID phenomenon discussed above, where antigens injected into the anterior chamber are shown to induce a deviant form of immunity. The injection process itself disrupts the integrity of the tissue, making it difficult to extrapolate from these findings to the intact eye. Therefore, these experiments cannot address the question whether the healthy eye is able to tolerize to its resident antigens. Although the posterior segment boasts of an efficient blood-retinal barrier, sequestration in fact does not apply to the anterior segment. While the tight junctions between adjacent vascular endothelial cells lining the structures of the anterior segment do provide a blood-aqueous barrier preventing entry of large molecules from the circulation, this barrier is unidirectional. The aqueous fluid that is constantly produced by cells lining the anterior chamber drains from the eye through the trabecular meshwork and the canal of Schlemm into the blood (http://www.lab.anhb.uwa.edu.au/mb140/CorePages/eye/eye.htm#iris). However, there is no direct communication between the aqueous and vitreous humors, so the anterior chamber is unlikely to constitute a way for retinal antigens to find their way out of the healthy eye. Direct evidence that antigens expressed in the retina do not make a significant impact on the immune system comes from experiments with mice expressing β-galactosidase (β-gal) as a neo-self antigen in the eye, where it constitutes a target for uveitis following β-gal immunization. Analysis of immunological responses of mice where expression of β-gal was localized only to the eye, compared with controls where it was expressed both in the eye and in the periphery, failed to find evidence of tolerance to β-gal in the former situation, whereas there was ample evidence for tolerance and reduced susceptibility to β-gal-induced EAU in the latter (59).

Two pieces of evidence might appear to challenge the interpretation that autoantigens in the posterior segment of the healthy eye are functionally sequestered. (i):In the β-gal Tg mice mentioned above, which express β-gal in the retina under control of the S-antigen promoter, Gregerson and Dou (60) reported reduced responsiveness to β-gal only under conditions of immunization that did not induce EAU (immunization in incomplete Freund's adjuvant). This result differed from their previous observation under uveitis-inducing conditions. They demonstrated transferable Tregs that could suppress DTH and found alterations in the cytokine profile, which, notably, were different from the pattern seen in ACAID. Based on these results and their inability to detect expression of β-gal in the thymus, they proposed that this constitutes evidence for eye-derived regulation. However, the S-antigen protein itself driven by its promoter is expressed in the thymus, so inability to detect β-gal driven by the same promoter could be due to insufficient sensitivity of the detection method, and they did not perform thymic transplants or depletion of $CD25^+$ cells to rigorously exclude central tolerance and nTregs. Experiments are required with mice having differential expression of retinal antigen in thymus versus the eye, possibly combining genetically engineered mice with thymic transplantation and/or enucleation to remove the source of ocular antigen. The contribution of the pineal gland (third eye) as a source of peripheral tolerogen will also have to be factored in. Irrespective of whether it is experimentally confirmed, a level of peripheral tolerance detectable only under non-uveitogenic conditions would have to be considered marginal. (ii) We have observed that IRBP-sufficient mice that are lethally irradiated, grafted with an IRBP KO thymus, and reconstituted with IRBP KO bone marrow (in which the only source of IRBP is the eye/pineal) regenerate a repertoire that is less responsive to IRBP than do their IRBP KO counterparts grafted with a KO thymus (46). However, while this finding demonstrates that peripheral tolerance can be induced in the absence of a thymic source of IRBP, it does not prove that the intact eye elicits such tolerance, because changes in the blood-retinal barrier resulting from the high-dose irradiation may have altered the accessibility of the ocular compartment. Experiments are needed that will address

this issue without altering the integrity of the eye. In the aggregate, data available thus far fail to provide support for a significant level of peripheral tolerance to antigens residing in the intact eye.

Immunosuppressive ocular environment impedes acquisition and expression of effector function but can be bypassed by activated effector T cells

The profoundly immunoinhibitory environment inside the eye and the paucity of MHC class-II-positive APCs in the retina make it unlikely that EAU could be initiated by errant lymphocytes entering the eye that become primed *in situ* and initiate an inflammatory cascade. This idea is supported by a large body of data demonstrating inhibition of activation and induction of regulatory function in naive T cells by ocular fluids and cells [17, 19–23, reviewed by Streilein (2)]. Priming of autoreactive lymphocytes most likely takes place in the periphery following exposure to retinal antigens released from a traumatized and possibly infected eye (sympathetic ophthalmia) or exposure to a cross-reactive microbial mimic (*Fig.* 2). Microbial mimics of S-antigen that induce EAU in rodents and in primates have been reported (61–63). Uveitogenic immunization in the periphery with retinal antigen emulsified in complete Freund's adjuvant is designed to mimic this hypothetical situation. Another method of introducing uveitogenic effector T cells into the body is by infusing cultured retinal-antigen-specific T cells that had been activated *in vitro* (42). An activated phenotype is critical for inducing EAU. T cells that have not been freshly activated are unable to find their way into the eye and induce EAU, possibly because they lack sufficient levels of expression of adhesion molecules, matrix-degrading metalloproteinases, and cytokines. The term 'homing' has been widely used to describe the process by which specific T cells find their target tissue. However, it is probably a misnomer when applied to these initial effector T cells infiltrating the healthy eye, because it implies some kind of specific attraction. It is hard to imagine how a migrating T cell can 'sense' that its antigen is on the other side of the blood-retinal barrier. Although mRNAs for some cytokines and chemokines can be detected in the healthy eye by real-time PCR (64), it is unclear whether they are expressed at the protein level. Our data indicate that the very first uveitogenic T cells find and enter the eye by chance (12). In the rat EAU model, fluorescently labeled activated lymphocytes from an S-antigen-specific uveitogenic T-cell line and concanavalin-A-stimulated 'non-specific' blasts initially enter the eye in the same numbers. These initial cells then disappear, and the eye appears quiet. If the cells are specific to S-antigen, several days later the eye develops destructive EAU, leading to the conclusion that EAU induction involves *in situ* antigen recognition. This notion has subsequently been confirmed by Thurau *et al.* (65) in the rat and by Chen *et al.* (66) in the mouse EAU model. It is currently not known on which cells this recognition might occur, as there are few or no MHC class-II-expressing cells in the uninflamed retina. It is plausible that these initial effector T cells, which are high IFN-γ producers (67), induce local expression of MHC class II on adjacent microglial or other cells, creating a microenvironment where antigen presentation can take place. Primed T cells have a lower threshold of activation than do naive T cells, and they are able to recognize their specific antigen under conditions that would be insufficient for *de novo* priming of naive T cells (68, 69).

How many activated effector T cells must enter the eye to induce EAU? We examined this question using Th1-polarized long-term T-cell lines, in which every cell should be a uveitogenic effector. In the study mentioned above, in which 10 million such Th1 cells specific to the major pathogenic peptide of S-antigen (freshly activated and labeled with the vital dye KH26) were infused into rats, about 150 cells were counted in the entire retina after 24 h (12). In a parallel experiment in the mouse, out of 5 million carboxyfluorescein-succinimidyl-ester-labeled cells from a Th1 cell line specific to the major pathogenic epitope of IRBP, about 75 cells were found in the retina after 24 h (S. B. Su and R. R. Caspi, unpublished data) (*Fig.* 3). Since only 1 million cells or less of these lines are needed to induce full-blown EAU, a simple calculation reveals that fewer than 15 retinal antigen-specific Th1 effector cells are sufficient to initiate the entire cascade of events that leads to clinical EAU. The entry of these cells into the eye requires intact Gi protein signaling, as treatment either of the cells or of the recipient animal with pertussis toxin (but not with the related cholera toxin, which inhibits Gs rather than Gi proteins) prevents their entry into the eye and causes them to accumulate in the circulation (*Fig.* 3). This finding implicates chemokine receptors, integrins, and other Gi-protein-dependent processes in the transmigration of these initial T cells entrants into the retina.

If such a surprisingly small number of activated effector T cells is sufficient to induce EAU, how effective is the local inhibitory environment in the eye to impede effector functions of incoming uveitogenic T cells and to prevent damage to vision? The emphasis here is on 'prevent'. As developed in the following section, systemic regulatory mechanisms that down-regulate EAU have been demonstrated, but they come into play only after tissue damage has already occurred and presumably are induced by antigens released as a result of tissue breakdown. The majority of published evidence on which the notion of local

inhibitory effects is based originates from *in vitro* studies in which ocular fluids or cells are brought in contact with T cells in culture. The aqueous humor can inhibit the function of effector T cells and induce Tregs from primed T-cell populations [reviewed by Taylor (24)], although it is not clear at the single-cell level whether a functional effector converts to a regulatory cell through this pathway. Although the vitreous cavity appears to elicit an ACAID-like phenomenon (36), information is lacking on the soluble mediators involved and how effective they might be to inhibit previously activated T cells. On the cellular level, retinal glial Müller cells and RPE cells in the back of the eye as well as ciliary body epithelial cells and corneal endothelial cells in the front of the eye have all been demonstrated to inhibit activation and function of previously primed mature effector T lymphocytes in culture (13, 14, 70, 71). These findings suggest the potential for such interactions *in vivo*. In support of this notion are findings that chemically induced damage to Müller cells by the gliotoxic agent L-α-aminoadipic acid results in enhanced EAU susceptibility (72). In contrast, despite its prominent role in some aspects of immune privilege (18), in our hands FasL expressed on ocular cells had no protective effect against EAU (73). Significantly, TGF-β was ineffective in inhibiting polarized uveitogenic Th1 cells, although naive and recently primed uveitogenic T cells were inhibited. Thus, highly activated mature effector T cells might be able to resist inactivation by the inhibitory ocular environment.

In eyes with uveitis, clearly the inhibitory threshold of the ocular environment has been passed; the effector T cells have gained the upper hand, and the inhibitory environment is altered. MHC class II expression is induced on retinal tissues, and there is influx of APCs from the circulation, which by themselves appear to be sufficient to support local antigen presentation in EAU (74). In eyes with EAU, immune privilege, as defined by the ability to induce ACAID and ability of the aqueous humor to inhibit T-cell activation, is temporarily lost (36, 75). This loss might permit *de novo* T-cell priming in eyes with uveitis. Priming of naive T cells in inflamed central nervous system tissue has been described and leads to the phenomenon of epitope spreading (76). It remains to be demonstrated whether such priming also occurs in the eye, but epitope spreading has recently been described in equine recurrent uveitis (77). Interestingly, aqueous humor that lost its ability to suppress T-cell activation still contained large quantities of TGF-β, and its ability to suppress was restored when IL-6 was neutralized (78). The recent reports that TGF-β in combination with IL-6 drives priming of Th17 effector cells (79, 80) raise the intriguing possibility that the right conditions for *de novo* priming of Th17 effector cells may exist within the inflamed eye.

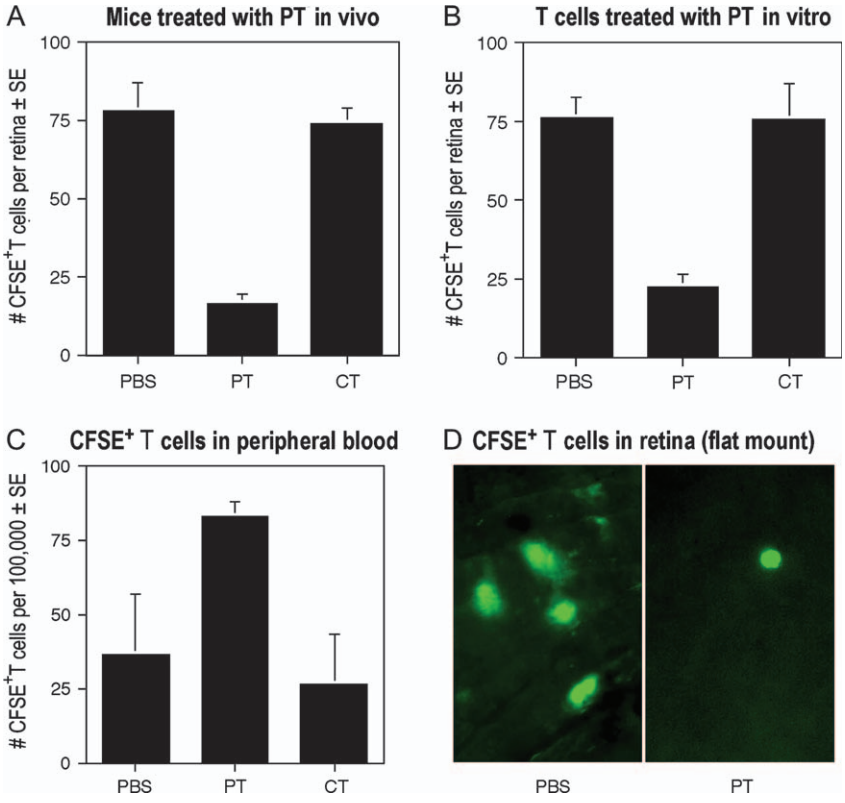

***Fig. 3.* Entry of activated uveitogenic T cells into the healthy eye is infrequent and requires Gi protein signaling.** (A) Five million carboxyfluorescein-succinimidyl-ester (CFSE)-labeled cells from a uveitogenic T-cell line were infused intravenously into recipients who were concurrently injected with pertussis toxin (PT), cholera toxin (CT), or phosphate buffered saline (PBS) for control. (B) Alternatively, the T cells were incubated with PTX, CT, or PBS *in vitro* for 1 h before transfer and were infused into untreated recipients. After 24 h, the eyes were collected, and individual retinas were dissected and flat mounted on microscope slides. (D) $CFSE^+$cells in the entire flat mount were enumerated by direct counting under a fluorescent microscope. (C) $CFSE^+$cells in peripheral blood were counted by flow cytometry. Four retinas (A, B) or five mice (C) were averaged for each point. (Figure by S. B. Su, Laboratory of Immunology, NEI.)

Active eye-derived tolerance mechanisms help restore immune homeostasis: a case of 'too little, too late'?

Although there is currently no compelling evidence for preexisting ACAID-like tolerance to antigens residing in the intact eye, once the blood-retinal barrier has been broken and antigens are released, such mechanisms could well come into play. The EAU process can be prevented if ACAID to IRBP is induced by injecting soluble IRBP into the anterior chamber of mice that are subsequently challenged with a uveitogenic regimen of this protein. No less importantly, IRBP-ACAID elicits splenic regulatory cells that are able to reverse the disease process after it had already started (81). Induction of this type of regulation required an intact spleen. Although induction of 'classical' ACAID via the anterior chamber is able to ameliorate EAU, it is unclear whether it is representative of regulatory phenomena that are induced spontaneously as a result of EAU.

Eye-derived tolerance is in fact induced as a consequence of EAU. Taylor and colleagues (37, 82) demonstrated that animals that have recovered from EAU harbor in their spleens postrecovery regulatory cells that are absent in naive animals and whose generation is dependent on α-MSH and the α-melanocortin receptor 5. Generation of these regulatory cells was dependent on the presence of the eye, as enucleation early after immunization prevented their generation. The actual mechanism why the eye is required is not known, but it is conceivable that it serves as a source of tolerizing antigen or of tolerogenic antigen-bearing APC. It is also possible that T cells that find their way into the eye during EAU acquire a regulatory phenotype after being exposed to α-MSH and their cognate antigen in the ocular fluids. These postrecovery Tregs appeared distinct from those induced by classical ACAID, in that they were $CD4^+$ regulators that could suppress already primed effector T cells, unlike classical ACAID, where that function is reserved for $CD8^+$ Tregs. Furthermore, for reasons that remain to be elucidated, there was a requirement for antigen activation of these cells by spleen cells that themselves had to have originated from EAU-recovered animals. It is unclear where, in the eye or in the lymphoid tissues, the regulation that finally controls the autoimmune process is effected. Labeling and tracking of the regulatory cells will be needed to answer this question.

It seems therefore that by efficiently separating the eye from the immune system, ocular immune privilege on the one hand allows persistence in the periphery of untolerized retina-specific cells that appear, once activated, to bypass the local ocular defenses with relative ease. On the other hand, the regulatory circuits that are induced as a consequence of the disease process itself step in only after the proverbial horse has already left the stable; although they control disease and restore immune homeostasis, the damage has already been done.

In conclusion

Immune privilege affects ocular autoimmunity in negative and in positive ways. In the dialog between the eye and the immune system, successive layers of privilege are called upon sequentially as they are needed. An effective separation between the eye and the circulation, accomplished by an efficient blood-retinal barrier, makes the eye largely invisible to the immune system, and this separation is normally sufficient. If an errant self-reactive lymphocyte enters the eye, a profoundly inhibitory intraocular environment steps in to control it and to prevent any damage before it begins. If that fails, as it does in the case of autoreactive lymphocytes that have already acquired effector function, the 'big guns'—active regulatory circuits—must be called into play. It is proposed that the passive and local aspects of privilege, largely aimed at preserving ignorance, are the main line of defense that the healthy eye uses to keep itself separated and protected from the immune system. However, the price is persistence of retinal antigen-specific T cells that cannot be peripherally tolerized but are only kept under fragile control by thymic derived nTregs. Eye-specific regulatory circuits are actively induced by antigens released from the damaged tissue. They constitute the final line of defense that is activated when the blood-retinal barrier is breached, and strong measures are needed to limit the generation of autoaggressive T cells. The new balance that is established may curtail the autoimmune process and restore functional tolerance, but it comes only as a consequence of initial damage to the very tissues that it is designed to protect. These notions challenge the accepted dogma that immune privilege protects from uveitis and raise the possibility that ocular autoimmunity is an unavoidable consequence of privilege and the price for day-to-day protection from the more common infectious insults that threaten vision.

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