CHARACTERIZATION OF THE ROLE OF REGULATORY T CELLS IN A MURINE MODEL OF UVEITIS

By

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A

DISSERTATION

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CERTIFICATE OF APPROVAL

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Rule #3: Count something. Rule #4: Write something. Rule #5: Change.

- Atul Gawande, *Better*

Abstract

Background: In this dissertation, the role of regulatory T cells in influencing the course of uveitis in a murine model is described. Uveitis, inflammation of the intraocular environment, is a significant cause of visual impairment. Regulatory T cells are a naturally occurring, immunosuppressive population whose role in uveitis remains to be elucidated. We hypothesize that regulatory T cells act in the inflamed eye to contribute to the resolution of uveitis in a murine model. **Methods**: The main technique used to evaluate the uveitis is non-invasive, epifluorescent intravital microscopy of the iris, and the principal parameters evaluated are onset of arrival, overall numbers, and time of disappearance of effector and host cells. The uveitis model involves adoptive transfer of fluorescently-labeled, antigen-specific effector T cells (DO11.10) to a congenic recipient animal harboring differently fluorescent $Foxp3^+$ regulatory T cells and/or $CD11c^+$ dendritic cells. The effect of regulatory T cell absence on effector cell behavior, as observed via intravital microscopy, is achieved through use of Foxp3-DTR animals whose regulatory T cells can be depleted through administration of diphtheria toxin. **Results**: The principal findings of this study are that iris-infiltrating effector T cells and $CD11c^+$ dendritic cells in our uveitis model are not observed to exhibit a statistically significant change in the observed parameters in the absence of systemic regulatory T cells. Other novel findings include: 1) regulatory T cells are not visible at baseline in the anterior segment of the naïve murine eye, including iris, cornea, aqueous humor, ciliary body, except in the limbal vessel arcade of the peripheral cornea. The cells accumulate at the limbal vessel arcade, in contrast to the earskin, in an age-dependent manner, and first appear in both tissues at fourteen days of age. Regulatory T cells were observed in far greater numbers in the palpebral conjunctiva of mice than in the bulbar

conjunctiva, which has not been described previously in rodents nor in humans. 2) Regulatory T cells and effector T cells were both observed in high numbers at early time points in the iris of the specific antigen-challenged eye, whereas few were present in the contralateral eye challenged with control protein. These two cells exhibited a consistent ratio of approximately 2-3:1 (effector: regulatory) during the first 48 hours after antigen challenge, before both cell populations decreased significantly. 3) $CD11c^+$ dendritic cells were observed to exhibit very little day-to-day change in their distribution in the irides of previously unmanipulated animals. However, they would exhibit significant change in response to inflammatory stimulus, both in the experimental eye and, to a much lesser degree, in the untouched, contralateral ("sympathizing") eye. 4) Iris-resident $CD11c^+$ dendritic cells were not observed to take up fluorescently labeled antigen, as determined by double-labeling using intravital and confocal microscopy, in contrast to previous reports. Instead, iris-resident Lysozyme M^+ phagocytic cells were observed to take up the labeled antigen, although their cells numbers did not change in response to antigenic challenge. **Conclusions**: Our results do not provide evidence of a role of regulatory T cells in contributing to the resolution of uveitis in our uveitis model, as determined by intravital microscopy of effector cell infiltration. This stands in contrast to other uveitis models, and may reflect model-specific differences. Notably, we use activated effector T cells in an adoptive transfer approach, which bypasses potential regulatory T cell control in the draining lymph node, where significant regulatory T cell activity has been noted in other uveitis models. This suggests that in self-limiting uveitis, the innately anti-inflammatory intraocular environment may be able to control inflammatory T cell activity without the contribution of regulatory T cells. Further investigations of the eye-intrinsic anti-inflammatory mechanisms of our model are

warranted. The novel finding of regulatory T cells residing specifically in the limbus suggests a possible role of these cells in contributing to corneal immune privilege.

Introduction

Chapter 1: Ocular Immunology

The first clues suggesting a unique immunological status for the eye date to Antiquity, when Hippocrates (c. 460-370 B.C.E.) is reported to have noted that "[p]eople who have lost one eye from injury frequently become blind in the other eye" (1, Samuels 1952). This pathological state, which has been widely documented throughout modern times, would later be termed "sympathetic ophthalmia" by William Mackenzie in 1840, and has been attributed to an autoimmune response to previously sequestered retinal antigens (2, Albert 1989). Further early evidence of the eye's unique immunological characteristics was demonstrated by the prolonged survival of allografts in the anterior chambers (ACs) of dogs and rabbits, in addition to similar experiments with xenografts and inanimate objects (3, van Dooremaal 1873). These observations would later be rigorously examined in comparison to grafts at cutaneous sites, ultimately resulting in the recognition that "grafts transplanted to the eye [] do not elicit an immune reaction," a finding attributed to the absence of lymphatics and later termed "immunological ignorance" (4, Medawar 1948; 5, Niederkorn 2003).

The molecular basis of ocular "immune privilege," as the modern understanding of "immunological ignorance" has come to be known, can conceptually be divided into three components: anatomical, local environment, and cellular (systemic) (6, Zhou 2010). In the first, the eye is effectively isolated from the vascular system by tight endothelial junctions that form the blood-ocular barrier, preventing the movement of large molecules and cells into and out of the healthy eye (7, Niederkorn 2007). Paired with the widely accepted notion of the general paucity of lymphatics in the mature, healthy eye, and the resultant absence of migrating dendritic cells bearing ocular antigens (8, Dullforce 2004), the immune system is effectively "ignorant" of

eye-derived antigens during normal homeostasis. In the second component, the intraocular environment is bathed by a panoply of anti-inflammatory molecules and immunomodulatory neuropeptides that squelch the inflammatory potential of infiltrating immune cells. Fas ligand (FasL), an important molecule that induces the apoptosis of Fas-bearing activated immune cells, is present on the luminal surface of intraocular parenchymal tissues, specifically the cornea, iris, ciliary body and retina (9, Griffith 1995). The neuropeptides α-melanocyte stimulating hormone (α-MSH), vasoactive intestinal peptide (VIP), somatostatin, and calcitonin gene-related peptide (CGRP) are all present at high concentrations in the aqueous humor (AH), where they have collectively been described as promoting an intraocular immunosuppressive environment by inhibiting immune cell proliferation, activation, and cytokine or effector molecule production (10-13, Taylor: 1992; 1994; 2003; 1998). TGF-β, a pleiotropic immunomodulatory molecule, is present in the AH in a soluble and cell-associated form, where it has been reported to exert an inhibitory effect on antigen-presenting cells (APCs), leading to decreased cytokine production and T cell activation (14, Taylor 1999). In addition to these soluble factors, surface-bound MHC class Ia are present at low levels on ocular parenchymal cells, whereas inhibitory Ib proteins are upregulated (15, Niederkorn 1999). Taken collectively, these molecules have the effect of presenting infiltrating immune cells, be they T, macrophage, dendritic, neutrophil, or NK, with an environment that is strongly predisposed to oppose their potential inflammatory function. The third component of ocular immune privilege, and perhaps the most complex and fascinating, involves a cell-based, eye-specific phenomenon resulting in systemic tolerance to ocular antigens, termed ACAID (anterior chamber-associated immune deviation). Historically, it was first demonstrated that the introduction of allotypic cells into the AC of rodents led to increased survival of subsequent cutaneous, corresponding allografts (16, Kaplan 1977); these findings

were eventually extended to tumors and numerous other foreign proteins, manifesting as a systemic downregulation of delayed-type hypersensitivity to the alloantigen (17-18, Niederkorn: 1981, 2002). Although the exact cellular mechanisms underlying ACAID remain a matter of debate (19, Camelo 2005), no fewer than four organ systems (eye, spleen, thymus and nervous) have been implicated in its induction, culminating in regulatory T cell-mediated systemic tolerance to the offending antigen (20, Stein-Streilein 2008).

A fourth component of ocular immune privilege, whose importance and central connection to the other three has only recently begun to be recognized comprises the conversion of infiltrating effector T cells into regulatory T cells during the course of intraocular inflammation (uveitis). Indeed, it has been reported that at least three different, physiologically relevant mechanisms for this phenomenon exist in the eye, in addition to a fourth experimental one (7, Niederkorn 2007). In the first, owing to the unique anatomical limitations of the ability of peripheral immune cells to infiltrate the eye, T cells that succeed in achieving diapedesis into the uveitic eye from either the retinal or iris vessels engage in intimate contact with retinal or iris pigment epithelial cells (RPE, IPE), which bear numerous immunomodulatory molecules. Mechanistically, this encounter involves the binding of CD86 on IPE/RPE and CTLA-4 on T cells, leading to the transformation of the infiltrating T effectors cells into TGF-β-producing regulatory T cells (21, Sugita 2003; 22, Yoshida 2000). In the second, the numerous immunosuppressive agents in the aqueous humor, in particular α-MSH, act on infiltrating T cells to promote their conversion to regulatory T cells and inhibit Th1-mediated autoimmune uveitis (23, Taylor 2001). This effect is enhanced by the activity of latent TGF-β in the AH, which is proteolytically activated in the setting of uveitis by serum-derived proteases to promote Treg formation and restore ocular immune privilege (24, Namba 2002). The third mechanism relates

to the induction of systemic tolerance to ocular antigens through the ACAID phenomenon; while the infiltrating T cell itself is not, strictly speaking, converted to a regulatory T cell by ACAID, nor are they required for its induction, regulatory T cells are generated in the spleen following injection of foreign protein into the anterior chamber of the eye (25, Keino 2006).

Regulatory T cells, it should be known, comprise a heterogeneous collection of immunosuppressive cells, the best studied and perhaps most numerous and physiologically significant of which are the $CD4^+CD25^+Foxp3^+$ regulatory T cells, which shall be called simply "regulatory T cells" in this text. Foxp3 is a transcription factor whose dysfunction in mice and men results in systemic, early-onset, fatal autoimmune disease in the absence of immunosuppression and/or bone marrow transplantation, testifying to its central importance in the function of regulatory T cells. Furthermore, Foxp3 is widely believed to be expressed solely in regulatory T cells, and serves to identify their lineage. Two distinct populations of $CD4^+CD25^+Foxp3^+$ regulatory T cells exist: natural, and induced. Induced regulatory T cells, as mentioned previously, arise from T cells that are converted to regulatory T cells in the periphery owing to their encounter with antigen in a non-stimulatory environment. Natural regulatory T cells arise in the thymus owing to recognition of self-peptides with their T cell receptor (TCR), although with a binding affinity insufficient to cause their deletion (via a mechanism termed "central tolerance"). These natural regulatory T cells emigrate from the thymus after day 3 of life (in mice) and migrate to secondary lymphoid organs and peripheral non-lymphoid organs, where they engage in maintaining peripheral tolerance to self-antigens. Regulatory T cells exert their anti-inflammatory effect through two general mechanisms: elaboration of immunomodulatory mediators, such as TGF-β, and through direct cell-cell contact. The cellular targets of regulatory T cell action can be found in both secondary lymphoid organs, such as draining lymph nodes and

the spleen, or in the non-lymphoid peripheral organs. In the secondary lymphoid organs, regulatory T cells are believed to exert their greatest systemic effect via interaction with dendritic cells, which are the principal and most effective antigen-presenting cells in the body and are generally characterized as expressing the adhesion molecule CD11c. In most organs, dendritic cells migrate through the tissue in a homeostatic manner, gathering self-antigens and promoting tolerance to them in the draining lymph node. However, in the setting of an immune response, dendritic cells are activated and promote the activation of T cells in the draining lymph node through the interaction with and presentation to the T cells of their cognate peptide and co-stimulation molecules. Regulatory T cells can modulate this event in the draining lymph node by suppressing the activation of dendritic cells through direct cell-cell contact, thereby preventing the activation of T cells by the dendritic cell. Regulatory T cells also act in the target tissue by interacting directly with effector (activated) T cells; mechanistically, regulatory T cells can inhibit the inflammatory activity of activated T cells by promoting their apoptosis or through other mechanisms, such as the consumption of required cytokines. In order to carry out this function, regulatory T cells have been observed to traffic through peripheral organs much in the same manner as dendritic cells, prior to migrating to draining lymph nodes via lymphatic vessels. It has recently been demonstrated that regulatory T cells can actually participate in and promote the immune response to pathogens and tumors, putting a new twist on their role as regulators of immunity.

As noted previously, the eye occupies a unique status vis-à-vis the immune system because it is relatively sequestered from immune surveillance owing to: a general lack of lymphatics, no dendritic cell migration from the organ, and the presence of tight endothelial barriers that prevent leukocytes from entering the eye during normal, healthy conditions. Given

these immunologic particularities, it is uncertain to what extent natural regulatory T cells are able to contribute to the maintenance of normal eye health as well as the recovery of ocular health after an inflammatory insult. In this text, we describe our efforts to illuminate the function of natural regulatory T cells in the eye, in settings of both health and disease.

Chapter 2: *In Vivo* Imaging of the Immune Response in the Eye

Intravital microscopy, the study via microscopy of biological events in living organisms, has tremendously advanced the analysis of the immune system in vertebrates (26, Gross 2007). This is especially true since the advent of the two-photon fluorescent confocal microscope, which has popularized *in vivo* imaging by combining single-cell spatial resolution with the ability to penetrate through superficial tissue (27, Sumen 2004). Given the dynamic nature of the immune system, wherein direct intercellular interactions between a cell and other cells or its environment are crucial for determining the course of immunologic events, it not surprising that many fundamental immunologic phenomena can not be examined faithfully outside of the host organism (28, Germain 2005). Examples of processes that are being studied in live animal systems include immune cell trafficking (29, Castellino 2006), intravascular migration (30, Auffray 2006), endothelial adhesion (31, Baatz 1995), activation (32, Miller 2004), suppression (33, Mempel 2006), antigen recognition (34, Hugues 2007) and maturation (35, Junt 2007a). Recent efforts by von Andrian and colleagues have demonstrated the power of this methodology when applied to the study of the immune system through multiple discoveries about the molecular and cellular events involved in effector and antigen-presenting cell (APC) co-interactions in the lymph node (36, Junt 2007b); these findings have been enriched by reports from Nussenzweig and Cyster utilizing *in vivo* imaging to examine the dynamic structure of

germinal centers (37, Schwickert 2007; 38, Allen 2007). The Lefrançois research group has achieved similar findings in the context of systemic bacterial infections by examining antigen-presentation in the spleen (39, Khanna 2007). Additionally, Bluestone and colleagues have demonstrated the versatility of this technical approach by shedding light on the role of regulatory T cells in the onset of autoimmune diabetes (40, Tang 2006). It is clear from these groundbreaking publications and many others that in the years to come, the use of intravital microscopy will help illuminate the cellular interactions that form the foundation of immunology.

All of the significant advances cited above, with one exception, involved the surgical excision or exposure of the target tissue, with the concomitant risk of introducing experimental artifacts; the one exception comprised research focused on cell migration in the eye (Baatz, 1995), an approach that has been reproduced elsewhere (41, Thurau 2004; 42, Becker 2000). Owing to its unique optical characteristics, the eye represents a remarkable opportunity for the analysis of the immune system by intravital microscopy (43, Rosenbaum 2002a). It is the sole organ that permits the passage of light in a physiologic manner without surgical intervention or other manipulations, and thus can allow for the observation of the interstitium of peripheral tissue and vascular beds in the absence of external interventions. Moreover, despite the complex anatomical nature of the eye, structures and vessels from multiple regions of the eye (e.g., sclera, cornea/anterior chamber, iris, retina/posterior segment) can be readily visualized. From an immunologist's perspective, this provides a model organ that allows for the unadulterated examination of the interaction of immune cells with each other as well as with multiple unique vascular beds and the extracellular environment (44, Rosenbaum 2002b). Additionally, the eye itself is subject to significant immunoregulation owing to its status as an immune-privileged

organ (45, Caspi 2006). The immune privilege of the eye results from multiple factors including a lack of lymphatics within the eye (46, Camelo 2006), expression of apoptosis-inducing factors such as Fas ligand (9, Griffith 1995), immunosuppressive factors in the aqueous humor like transforming growth factor beta (47, Cousins 1991), and avascularity of the healthy lens and cornea (48, Cursiefen 2002). Despite these features that make broad extrapolations tentative, the eye is a frequent target of many immune-mediated diseases (49, Smith 2002). For these reasons, there has been significant interest and progress in the study via intravital microscopy of the molecules involved in leukocyte trafficking in the eye (50, Becker 2006), a key process in the initiation of both eye-specific and systemic immune-mediated diseases (51, Ley 2007). We present a brief overview of the current state of knowledge of leukocyte trafficking in each of three anatomically distinct and medically important regions of the eye (cornea, iris, retina) as determined by the application of intravital microscopy to animal models of disease. Additionally, we discuss the use of intravital microscopy on human subjects. Finally, we examine the future prospects for this field in terms of its potential for impacting our understanding of fundamental immunological phenomena as well as aiding in the development of novel therapeutic agents.

In addition to intravital microscopy, other technologies such as PET (positive emission tomography), MRI (magnetic resonance imaging), ultrasound, luminescence, and OCT (ocular coherence tomography) can help to image aspects of inflammation of the immune response. Our discussion here is primarily devoted to microscopy which thus far is the technique that has the best resolution to identify individual cells.

Imaging of leukocyte migration in the iris

The study of the iris by intravital microscopy presents an exceptional opportunity for visualizing the immune response *in vivo*, owing to its unique anatomical features, optical

properties, and direct involvement in models of immune-mediated diseases. The iris is situated immediately posterior to the cornea and anterior chamber in a plane perpendicular to ambient light originating external to the eye. Aside from enabling the iris to regulate the amount of incident light arriving at the retina, this location also allows for direct, non-invasive visualization of the iris parenchyma with a standard epifluorescent microscope, obviating the need for a two-photon microscope. Moreover, as the iris lies in a relatively flat plane, the identification and tracking of anatomical features and cells is straightforward. Additionally, the murine iris is a thin tissue consisting of only a few cell layers in thickness and, in laboratory animals lacking pigmentation, is translucent, facilitating the study of cellular activities. During states of inflammation, both intravascular and extravascular leukocytes are readily observable in the vessel-rich iris. A single episode of iris-associated inflammation may be followed throughout its course at multiple timepoints due to the non-invasive nature of the technology. Thus, as a component of the uvea, the anatomical region comprising the iris, ciliary body and choroid, the inflamed iris serves as a useful proxy to enable the study of the immune-mediated disease uveitis, a major cause of blindness and morbidity (43, Rosenbaum 2002a).

The uveal tract, like the brain, lacks lymphatic vessels. An accepted dogma in immunology is that the local injection of an antigen induces the migration of antigen presenting cells from the site of immunization to a local lymph node. The laboratory of McMenamin and others as well as our own has shown that the uveal tract contains an abundant network of potential antigen presenting cells (52, Steptoe 2000; 53, Meng 2007). How can these cells reach a regional lymph node without a lymphatic channel to guide them? Intravital studies by Dullforce and colleagues indicate that the vast majority of APCs within the iris are totally stationary even when stimulated with both antigen and endotoxin (8, Dullforce 2004). This

correlates with an inability to detect fluorescent beads in a regional lymph node after these are injected into the anterior chamber. In contrast, a soluble antigen can move from the anterior chamber to a local node, presumably via a uveal scleral outflow tract or through the trabecular meshwork. Streilein, Niederkorn and others have characterized a suppression of a cell-mediated immune response when a soluble antigen is injected into the anterior chamber (54, Streilein 1981), a phenomenon known as ACAID (anterior chamber-associated immune deviation). Jenkins and colleagues have likewise shown a suppression of the immune response when antigen is delivered directly to a lymph node rather than carried there by an antigen presenting cell (55, Kearney 1994). Although ACAID is a complex phenomenon, the stationary nature of iris APCs may well be a major contributor to it.

Experimental ovalbumin-induced uveitis (EOU), the murine model of uveitis currently most studied in our laboratory, is a form of experimental uveitis that is mediated by antigen-specific infiltration of $CD4^+$ T cells into the iris, closely mimicking the pathology of the immune-mediated form of the human disease (56, Dullforce 2006). After passive transfer of *in vitro* cultured ovalbumin-specific $CD4^+$ T cells, anterior chamber (a.c.) challenge with ovalbumin (OVA) results in a brisk infiltration of inflammatory cells including OVA-specific $CD4^+$ T cells and host-derived neutrophils that peaks at 24 hours before subsiding within a few days. Through the use of a combination of cell-staining fluorescent dyes and fluorescently-labeled antigen (57, Becker 2003), we have been able to exploit this system with a standard epifluorescent intravital microscope to analyze the trafficking and intercellular interactions of leukocytes in the inflamed iris. A major goal of this research is to identify the surface molecules involved in these processes, including integrins and chemokine receptors. In the gut and skin (58, Kunkel 2002), unique combinations of these molecules designate tissue-specific lymphocyte

subsets that home preferentially to those tissues drained by their lymph node of origin; much of this information has been generated by intravital microscopy of lymphoid tissues during the maturation phase of the immune response (59, Mebius 2007). In this regard, this EOU model is unique in that allows the direct observation via intravital microscopy of leukocytes at a site of inflammation, thus enabling the characterization of molecules involved in regulating the effector stage of disease. Specifically, we have been able to identify and characterize cell types and molecules involved in trafficking to the inflamed iris, as well as interactions with the activated endothelium and antigen-presenting cells; these findings might have extrapolations for understanding leukocyte migration in other organs.

In this model, we observed that the infiltrating lymphocytes were predominantly antigen-specific by comparing the number of OVA-specific T cells with hemagglutinin-specific T cells (HNT) after ovalbumin, non-specific protein or saline challenge in the a.c. In this setting, both sets of T cells were labeled fluorescently with carboxyfluorescein succinimidyl ester (CFSE), and infiltrating cells were observed via intravital videomicroscopy. While extravascular CFSE-labeled cells were not detected in the anterior chamber, antigen-specific labeled cells accumulated in the conjunctiva, limbus, ciliary body and iris. Although the movement of extravasated T cells was too slow to capture via real-time videomicroscopy, their accumulation was readily tracked via imaging at multiple timepoints (6, 24, 48, 72 hrs) (56, Dullforce 2006). We further showed that there was preferential accumulation of memory $CD4^+$ T cells, characterized by their $CD44^{high}$ phenotype, compared to naïve $CD44^{low}$ cells. Examination of the effect of the T-helper subtype (Th1 vs. Th2) was also pursued, as evidence suggested that the Th1 cells were more uveitogenic (60, Tarrant 1998). *In vitro* differentiated Th1 and Th2 subsets were transferred to OVA-challenged animals, and the Th1 cells accumulated at significantly

greater levels than Th2 or undifferentiated effector cells. Additionally, given the data suggesting a central importance for integrins in neutrophil adhesion to activated endothelium in an endotoxin-induced uveitis model (61, Becker 2001), we analyzed the role of surface adhesion molecules on T cell trafficking to the iris in this model. One promising candidate was α_2-integrin, which had been implicated in the trafficking of antigen-specific CD8+ T cells to a site of inflammation, and as a component of the adhesion molecule VLA-2 has been shown to bind extracellular matrix components (62, Andreasen 2003; 63, Elices 1989). Indeed, we noted that the $CD44^{high}$ T cells that caused significant uveitis in our model expressed α_2-integrin at a high level, in contrast to the non-uveitogenic $CD44^{low}$ cells. Blocking of α_2-integrin or transfer of α_2-$integrin^{low}$ T cells resulted in decreased T cell accumulation in the iris compared to control. Anti-α_2-integrin antibody was noted to inhibit arrival of *in vitro*-activated Th1 cells, suggesting a role for this surface molecule in the adhering of memory T cells to activated endothelium.

In addition to using intravital videomicroscopy to study the influence of effector status, Th-subtype, and integrin expression on the trafficking and uveitogenic potential of $CD4^+$ T cells, we have also examined the interaction of effector cells with antigen-presenting cells (APCs). This interest stems in part from the observation that, during acute uveitis, a significant number of labeled T cells had extravasated into the iris tissue and appeared to be in close proximity to fluorescently-labeled, major histocompatibility complex (MHC) class II^+ APCs. As both naïve and effector T cells were observed to accumulate at early time points in the EOU model in an antigen-independent manner, but antigen-specific cells dominated thereafter, we considered the possibility that increased survival or retention of these cells may have resulted from interactions with APCs bearing the appropriate peptide/MHC complex, as suggested by recent publications (64, Reinhardt 2003). Using intravital videomicroscopy, we chose to investigate the existence of

such an encounter and characterize the dynamics of the T cell migration that it impacted (65, Rosenbaum 2008). In the presence of cognate antigen and lipopolysaccharide, labeled effector cells accumulate in the iris vessels and extravasate into the stroma. Using time-lapse videomicroscopy to image the extravascular movements, the effector T cells were observed to migrate throughout the stroma in a manner consistent with a random walk at a speed of 1-2μm/min, whereas the fluorescently-labeled APCs remained stationary. In the absence of cognate antigen, although effector T cells would accumulate at significantly lower levels, those cells that did extravasate exhibited the same random migration profile as antigen-specific effector T cells, with similar motility. In contrast, OVA-specific effector T cells exhibited greater contact with APCs labeled with OVA-antigen than those bearing a control antigen. This preference for a specific antigen, however, was not confirmed when effector T cells of a different specificity (HNT) were exposed to OVA-bearing APCs. When compared with other studies of T cell migration *in vivo*, these data demonstrate that effector T cells at a site of inflammation migrate at a significantly slower speed and via a different migration pattern than that observed in lymphoid tissues (4-11 μm/min, directed) (66, Stoll 2002; 67, Witt 2005); these differences likely reflect as-yet-unknown distinct characteristics of the extracellular matrix upon which the cells navigate as well as the adhesion molecules that they employ to do so. Furthermore, these experiments exemplify the uniqueness of this experimental approach, wherein the dynamic interactions of leukocytes with APCs can be investigated *in vivo* in the context of an immune-mediated disease, and open the door to identifying molecules involved in this pathologic process.

Imaging of the immune response in the retina

The retina is one of the ideal tissues for imaging the immune response as it is possible to observe the blood vessels directly by using common equipment, such as a fundoscope. This advantage also can be applied to real time *in vivo* imaging of an experimental animal model. Confocal microscopy is usually used to observe the site of inflammation in the anterior part of the eye, such as the cornea and iris. It is technically more challenging to visualize clear images of the cell dynamics in the retinal tissue as it is located at the posterior part of the eye. Instead, scanning laser ophthalmoscopy (SLO) has the greatest capability to visualize these images in the retinal vasculature (68, Sharp 2004).

Numerous studies focusing on the *in vivo* imaging of the immune response in the murine retina have been published by Forrester and colleagues (69, Xu 2002a; 70, Xu 2004a; 71, Xu 2002b; 72, Xu 2004b; 77, Xu 2003; 79, Crane 2006). They have developed a novel model that enables them to assess the leukocyte dynamics within the retinal vasculature in living animal without surgical intervention, especially the interaction between inflammatory cells and retinal endothelial cells, such as rolling and sticking (69, Xu 2002a). In this model, sodium fluorescein is injected via tail vein to outline the retinal vessels, followed by injection of Calcein-AM-labeled syngeneic T cells. A hard contact lens is placed on the cornea to obtain a clear view, and the fundus is examined by the SLO system. Using this technique, the investigators could determine cell velocity and shear stress in the retinal vasculature of the normal B10.RIII and BALB/c mice under physiological conditions (69, Xu 2002a).

Experimental autoimmune uveitoretinitis (EAU) is a well-known, established model that has been instructive in elucidating uveo-retinitis (73, Forrester 1992; 74, Mochizuki 1985; 75, Silver 1995) and this model matches well with the SLO-guided cell tracking system. Various combinations of experiments are possible with this method in that cells from normal or

immunized mice can be transferred to normal mice or immunized mice in various stages of EAU. In order to produce inflammation in the retinal tissue, inflammatory cells, activated autoreactive T cells in this case, need to accumulate in the eye, adhere to the retinal endothelium and transmigrate into the retina across the blood-retina barrier (BRB). These phenomena of inflammatory cells are preferentially seen at postcapillary venules and veins in EAU and are strongly related to hydrodynamic factors. This model has revealed that shear stress in the retinal vein was reduced 24 hours before cell infiltration. In addition, this reduction was negatively correlated with the number of rolling and sticking cells (70, Xu 2004a).

These cellular interactions in the retinal vasculature are mediated by cell-adhesion molecules similar to those seen at the site of inflammation in other organs. CD44, P-selectin glycoprotein ligand 1 (PSGL-1) and lymphocyte function associated antigen-1 (LFA-1) are reported to be up-regulated on activated $CD4^+$ T cells in EAU, whereas intracellular adhesion molecule 1 (ICAM-1), P-selectin and E-selectin are up-regulated on the retinal venules (76, Xu 2003). CD4 positive T helper (Th) cells have been shown to be polarized into two subsets, Th1 and Th2, characterized by their cytokine production. EAU is generally described as a Th1-type autoimmune disease (77, Sun 1997), with the contribution of Th17 cells now being clarified. As described above, the selection of the T cell subsets to be transferred offers the opportunity to analyze cell trafficking of specific T cell populations. Also pretreatment of cells to be transferred with blocking antibody to specific adhesion molecules can confirm the functions of these molecules in T cells. Moreover, subsequent confocal microscopy of retinal whole mounts allows correlation of *in vivo* findings with extravascular infiltrations. In EAU, the Th1 population showed much higher rolling and sticking efficiency in retinal vasculature than the Th2 population. In addition, the rolling velocity of Th1 cells was much lower compared with that of Th2 cells. These results

directly demonstrate that the Th1 population is preferentially recruited into the inflamed retina in EAU model.

With regards to cell-adhesion molecules, blockage of PSGL-1 suppressed the rolling of the Th1 but not Th2 population, whereas blockage of CD44 suppressed the rolling of both cell populations. In contrast, blocking of LFA-1 did not reduce rolling efficiency, although it resulted in a reduction in the number of both subsets infiltrating the retinal tissues. These data indicate that random recruitment of activated T cells appears to be mediated by CD44: CD44 ligand (hyaluronic acid) and LFA-1: ICAM-1 interaction, whereas the selective recruitment of Th1 cells is mediated by PSGL-1: P/E selectin (72, Xu 2004).

As with cell-adhesion molecules, much interest is focused on the role of chemokines that direct the migration of T cells. Pretreatment of Th1 cells to be transferred with antibody against CC chemokine receptor 5 (CCR5) reduces their infiltration into the retina, but not rolling and sticking, indicating that CCR5 is also required for Th1 cell recruitment in EAU and may act at the level of transendothelial migration rather than at the initial stage of cellular interaction (78, Crane 2006).

Summary and Future Prospects

The application of intravital and especially multiphoton microscopy to the study of the vertebrate immune system has yielded tremendous insight into the cell-cell interactions that lie at the heart of immunology. This is not surprising, as the immune response is a complex, multi-step, dynamic process that is continuously evolving within an organism, so the static "snapshots" of information provided by more conventional microscopy will always be incomplete. In this regard, the eye presents a remarkable opportunity for visualizing the immune response *in vivo*, as its optical, anatomical and physiological attributes render it highly conducive to the direct

visualization of numerous immunologic phenomena in an unperturbed experimental context. This may be best illustrated by studies on the cornea, which is an ideal tissue for imaging given its inherent transparency and superficial location. The dynamic behavior of multiple immune cells, including neutrophils, dendritic cells and T cells has proven to be highly amenable to characterization in this tissue using combinations of exogenous fluorescent labels and cell-type specific expression of fluorescent proteins. Similarly, the iris readily lends itself to the intravital imaging of immune cell dynamics, and owing to its rich vascular network, provides a useful model for the study of the immune cell interactions with activated endothelium. Furthermore, we have shown that the dynamics of extravasated T cells in the iris stroma and their interactions with APCs can be examined in the context of local inflammation, offering a means for evaluating the role of various molecules in mediating this biologically important event. The retina constitutes a more technically challenging object of study by *in vivo* imaging than the constituents of the anterior segment. With the use of appropriate tools and experimental models, such as the scanning laser ophthalmoscope and EAU, *in vivo* imaging of the immune response in the retina has yielded valuable insight into the molecular mechanisms of immune-mediated diseases afflicting the vasculature of the posterior segment.

Looking to the future, the prospects for research predicated on *in vivo* imaging of the immune response in the eye are bright. This is due in part to the development of new research tools, such as a wide-range of novel endogenous fluorescent proteins with unique spectral characteristics (79, Giepmans 2006). Coupled with an increasingly diverse gamut of available exogenous fluorescent molecules (80, Medintz 2005), the use of these agents will allow for the *in vivo* identification, trafficking and characterization of multiple cell types and their interactions in dynamic processes like the immune response (33, Mempel 2006). Using transgenic technology,

virtually any cell subset can be tagged for identification by microscopy. Furthermore, intravital microscopy can identify intracellular events such as the synthesis of interleukin-2 as a marker of T cell activation, wherein a fluorescent protein like GFP can be made dependent on a specific promoter using transgenic and molecular technology (81, Naramura 1998; 82, Becker 2001). In addition to the technology-driven advancements described above, growing recognition of the versatility of the eye as a model for studying the immune response by intravital imaging has increased the popularity of this experimental approach, leading to a greater range of topics being explored with this system. A key example is that of the recently described, inflammatory T helper cell subset, Th17, which has been implicated in uveitis (83, Amadi-Obi 2007). Using intravital microscopy of the EOU model, Zhang and colleagues have been able to demonstrate that Th17 cells are primarily responsible for neutrophil recruitment into the uvea in this model (84, Zhang 2009). Future experiments focused on characterizing these pathogenic cells through the use of this model will certainly be inspired by these results, as Th17 cells have a causal role in numerous other autoimmune diseases (85, Steinman 2007). Labeling of infectious agents could allow clarification of the mechanisms by which the immune system responds to pathogens in the eye. The accessibility of vessels in the human eye provides unique opportunities to quantify rolling and sticking and to measure the effect of pharmacologic agents on these phenomena. In summary, the use of intravital microscopy to visualize the immune response in the eye has enabled us to gain significant knowledge about leukocyte function *in vivo*, and is likely to illuminate many immunological and clinical fields in the future.

For several reasons, including those detailed above, we believe that our EOU model offers significant advantages compared to EAU for investigation via intravital microscopy of intraocular inflammation. First, and perhaps most importantly, the cellular infiltration in EOU is

readily visible in the anterior segment of the eye, as opposed to predominantly in the posterior segment in EAU. In addition to lending itself much more readily to visual analysis, anterior segment inflammation also is the most common form of human uveitis. In terms of the technical approach, the EOU model is advantageous in that the onset of cellular infiltration is occurs soon after intravitreal injection of antigen, whereas this occurs at fourteen after immunization in EAU models. We believe this occurs because, as stated previously, we adoptively transfer antigen-specific T cells from immunized animals, and we noted that it is the memory cells that preferentially accumulate (56, Dullforce 2006). *In vitro* activation has been noted empirically to contribute to the uveitogenic potential of the transferred T cells, likely owing to differentiation of these cells into Th1 cells (60, Tarrant 1998). This timeline corresponds with the delayed onset of disease in classical EAU models, and suggests a similar requirement for activated, memory cells in both models. It should be noted that our *in vitro* culture step likely replicates the lymph node maturation that occurs in the endogenous setting in EAU, which explains why adoptively transferred T cells traffic directly to the site of antigen injection in our model; this is supported by our observation that cells that bypass the *in vitro* culture step in our EOU model do not lead to significant iris infiltration (Mischa Ronick, personal communication). Further investigation of this event through comparison with trafficking of transferred cells to other sites of antigen injection, such as the skin, may shed light on the role of antigen persistence in contributing to the duration of inflammation, as the cellular infiltration observed in our model is short-lived compared to classical EAU, where the antigen is endogenous; in the eye, the intraocular fluid is replaced over the course of several hours through normal flow of aqueous humor, whereas antigen is likely to persist longer in the skin. We have also noted that uveitis onset is limited to three days after the T cell transfer, as intravitreal injection beyond this period does not result in

significant iris infiltration (Mischa Ronick, personal communication). This likely reflects the activated nature of the transferred T cells, as Th1-differentiated memory cells were shown to possess the greatest uveitogenic potential, but also express adhesion molecules that would limit their ability to persisit indefinitely in the circulation (62, Andreasen 2003; 63, Elices 1989). Through the adoptive transfer approach which our model enables, it would be possible to dissect the uveitogenic potential of T cells of various activation and differentiation subsets, which is more challenging although still feasible in a classical EAU model (86, Luger 2008).

Statement of Problem

It has been established by many authors that regulatory T cells can contribute to the resolution of uveitis in rodent models (see Discussion). However, the exact nature of this contribution remains undetermined. Whereas the immunosuppressive activity of regulatory T cells in the draining lymph node has been implicated in these studies, the contribution of regulatory T cells acting in the inflamed eye has not been investigated. For this reason, we set out to examine the potential anti-inflammatory effect of regulatory T cells acting primarily in the eye in the setting of our experimental ovalbumin-induced uveitis model. **We hypothesize that regulatory T cells are present *in situ* in the uveitic eye, and that these cells contribute to the control and resolution of uveitis through influencing the activity of infiltrating effector cells, including antigen-specific T cells and iris-invasive dendritic cells**. Using non-invasive, intravital imaging to interrogate our uveitis model, we postulate that we will be able to establish:

1) The baseline activity of regulatory T cells in the naïve murine iris. The presence (or absence) of regulatory T cells in the naïve murine eye has not been previously established, in contrast to other tissues. Given the widespread distribution of regulatory T cells in other

peripheral organs in homeostatic conditions, counterbalanced with the immune-privileged nature of the eye, it is unclear what the baseline presence of regulatory T cells will be in this tissue.

2) The baseline activity of regulatory T cells and effector T cells in the uveitic iris. The presence of regulatory T cells in the uveitic eye has only been investigated during the convalescent stage, in contrast to other tissues where they have been shown to be present in high numbers at early stages of inflammation. In light of the ability of effector T cells to invade the eye in our and other uveitis models, we anticipate that regulatory T cells will also be present in the inflammatory lesions.
3) The effect of regulatory T cell absence on effector T cell activity in the uveitic eye. Previous reports have investigated the influence of regulatory T cells on the clinical and histopathological score in uveitis, but not on effector T cell parameters (onset of arrival, overall numbers, and resolution) in the inflamed eye. Regulatory T cells have been shown to exert their immunosuppressive activity on effector T cells in other organs through action in the lymph node and in the inflamed tissue, although the latter has not been demonstrated in uveitis; we hypothesize that regulatory T cells influence effector T cell infiltration of the inflamed iris.
4) The baseline activity of dendritic cells in the naïve and inflamed murine iris. Dendritic cells have been shown to traffic through other tissues in homeostatic conditions in order to present tissue-specific self-antigen in the draining lymph node, although no evidence for this behavior has been demonstrated in the eye. In inflammatory conditions, dendritic cells have been recently described to invade the target organ in a manner that is controlled by regulatory T cells, as is well established with effector T cells. We anticipate that, in

parallel with effector T cells and despite the immune-privileged nature of the eye, dendritic cells will be observed to infiltrate the inflamed iris under the influence of regulatory T cell control.

Materials and Methods

Mice

T lymphocytes from DO11.10 mice have transgenic T cell receptors that specifically recognize $OVA_{323-339}$ peptide (ISQAVHAAHAEINEAGR) in the context of $I\text{-}A^d$, and females were used as T cell donors (Jackson Laboratories, Bar Harbor, ME). All animals used in adoptive transfer experiments were 6-12 weeks old; animals used in other experiments were at the ages specified. Foxp3-EGFP (BALB/c, C.Cg-*Foxp3*$^{tm2(EGFP)Tch}$; 87, Haribhai 2007) mice were used as T cell recipients (Jackson Laboratories, Bar Harbor, ME), as well as DEREG mice (Foxp3-EGFP-DTR, BALB/c; 88, Lahl 2011), which were generated and obtained from Dr. Tim Sparwasser (TWINCORE, Hannover, Germany). Mice expressing enhanced yellow fluorescent protein (EYFP) under the control of the CD11c promoter (CD11c-YFP; 89, Lindquist 2004), which is specific for dendritic cells, and mice expressing EGFP under the control of the Lysozyme M promoter (LysGFP; 90, Faust 2000), which is specific for macrophages and neutrophils, were crossed onto the BALB/c background for 10 generations at OHSU. CD11c-YFPxDEREG and Foxp3GFPxDEREG animals were bred at OHSU. The animal experimental protocols were in accord with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by our institutional animal care and use committee.

Preparation of CD4 T cell population, adoptive transfer, intravitreal injection/uveitis induction

At least 7 days before splenic harvest, donor DO11.10 mice were immunized with multimeric OVA peptide via intraperitoneal injection (20 μg; 91, Wegmann 2008). Single cell suspensions were prepared by passing the tissue through a 70 μm cell strainer (BD Biosciences, Mountain View, CA). Red blood cells (RBC) were lysed with 1X RBC lysis buffer (Sigma–Aldrich, St

Louis, MO) at room temperature for 5 min. The cell suspension was washed twice with RPMI 1640 (Invitrogen, Carlsbad, CA), and then cultured in RPMI 1640 with 10% fetal bovine serum (FBS) in an atmosphere of 95% air and 5% CO2 at 37 °C. The DO11.10 splenocytes (4×10^6/ml) were then cultured with $OVA_{323–339}$ peptide (Ile-Ser-Gln-Ala-Val-His-Ala-Ala-His-Ala-Glu-Ile-Asn-Glu-Ala-Gly-Arg) (AnaSpec, Fremont, CA) (5 μg/ml). T cells were then isolated using Lympholyte-M density-separation media (Cedarlane Labs, Ontario, CA), and incubated with the red fluorescent dye CMTMR (10μM; Molecular Probes, Eugene, OR) for 10 minutes in PBS. Red-labeled T cells were then injected intravenously into the tail vein of 6-12 week-old naïve BALB/c recipient mice ($2x10^7$ cells/animal). After i.v. tail injection, these mice were challenged with a 2 μl intravitreal (ivt) injection of 50 μg chicken ovalbumin (Grade V; Sigma Chemical, St. Louis, MO) and 200 ng *E. coli* strain lipopolysaccharide (LPS; Sigma) in PBS. Contralateral eyes were injected with equivalent amounts of a control protein (Bovine Serum Albumin (BSA); Sigma Chemical, St. Louis, MO) and LPS. For experiments involving adoptive transfer of DO11.10 T cells into CD11c-YFP^+ recipients, the LPS was not included in the intravitreal challenge mixture. For intravitreal injections not occurring in the setting of adoptive transfer experiments, mice were challenged with a 2 μl of 10 μg/μL of Alexa Fluor (AF) 594 (AF594, Molecular Probes, Eugene, OR) (red)-conjugated chicken ovalbumin (OVA) (Grade V; Sigma Chemical, St. Louis, MO) in PBS. CD11c-YFP^+ or $LysGFP^+$ cells were considered AF positive based on overlap of fluorescence in intravital images using a red-green dual filter.

Intravital imaging and confocal microscopy

At various time points following intravitreal challenge, fluorescent host and adoptive T cells were imaged in mice anesthetized with inhalation of oxygen (1 l/min) and isoflurane (2%;

Ohmeda, Liberty Corner, NJ), using a modified DM-LFS epifluorescent Leica microscope (Orthoplan, Leica, Wetzlar, Germany) and an an Optronics DEI color camera (Goleta, CA). Magnification of still images was 200x. A gel, GenTeal (Novartis, East Hanover, NJ), was applied between the cornea and an aqueous immersion 20x Leica objective. LabView 8.5 (National Instruments, Austin, TX) was used to interface with and acquire images using customized Matlab software (Mathworks, Natick, MA). The irises of naïve Foxp3GFP, CD11cYFP and LysGFP animals were imaged at the specified time points. For *ex vivo* confocal microscopy, irises were dissected from enucleated eyes after fixation in 4% (w/v) paraformaldehyde (four hour to overnight) and then imaged using an Olympus Fluoview FV1000 laser scanning confocal microscope. Images were taken at 1µm slices.

Antibodies and flow cytometry

DO11.10 splenocytes (1-5x10^6) were suspended in PBS containing 2% FBS and 0.1% sodium azide. Using all eBioscience reagents per the manufacturer's instructions (San Diego, CA), FITC-conjugated anti-CD4 (clone GK1.5) and APC-conjugated anti-CD25 (clone PC61.5) antibodies were used to label these cell surface markers for 30 minutes at 4 °C. After PBS wash, the cells were fixed with 1× fixation solution for 20 minutes at room temperature. Cells were then incubated in permeabilization buffer with PE-conjugated anti-Foxp3 (clone FJK-16s) for 60 minutes at 4° C. After a PBS wash, data acquisition was performed on a FACS Calibur flow cytometer and data were analyzed using CellQuest software (BD Biosciences, Franklin Lakes, NJ).

Foxp3-DTR genotyping, ablation and flow cytometry

Foxp3-GFP-DTR (DEREG) mice were genotyped for DTR by PCR using the primers P442 (5′-ccc agg tta cca tgg aga ga -3′) and P443 (5′- gaa ctt cag ggt cag ctt gc -3′) (92, Lahl 2007). The reaction conditions took place in 25 μL in the presence of 100 μM of each primer and 50 ng of genomic mouse DNA. The amplification conditions were the following: 94°C, 1 minute; 35 cycles of (94°C, 1 minute; 60°C, 1 minute, 72°C, 1 minute); 72°C, 10 minutes. The specific band migrated around 350bp, and was the most prominent band in positive samples. Positive and negative controls were used for each experiment due to the presence of multiple non-specific bands (Figure 18). Ablation of Foxp3-DTR T cells was achieved through the intraperitoneal injection of 1 μg of once-thawed, individually-aliquoted diphtheria toxin (< 6 months old; Sigma-Aldrich, St. Louis, MO) in PBS on days 1 and 2, with mice considered Foxp3-DTR depleted on day 3. Foxp3-GFP-DTR absence was confirmed via flow cytometry of peripheral blood (50 μL; Figure 15), evaluating for GFP positivity, and intravital microscopy, assessing for the absence of GFP^+ cells in the uveitic murine limbus on days 4-5 (after uveitis induction on day 3); GFP^+ cells would be present in the PBS-injected control Foxp3-GFP-DTR animals at 24 hours after uveitis induction, and would reappear in DT-treated animals starting on day 6 (Figure 14).

Statistics

Images of fluorescent cells were quantified in a masked manner by a third party. Unpaired two-tailed Student's t-test was performed (Prism Software, Irvine, CA) to determine the statistical significance (*$p<0.05$).

Results

Foxp3-GFP$^+$ cells are present only in the limbal region in naïve murine eyes. In order to undertake an initial characterization of the role of regulatory T cells in the healthy murine eye, we set out to determine the distribution of Foxp3-GFP$^+$ cells in the anterior segment of naïve eyes in the transgenic Treg reporter animal, BALB/c Foxp3-GFP, via non-invasive intravital epifluorescent microscopy. Naïve, healthy-appearing male and female animals were examined at a single time point per animal, either at weaning age (18 days) or as adults (12 weeks), in order to consider the possibility of an effect of age on the ocular distribution of these cells. Foxp3-GFP$^+$ cells were observed bilaterally in the limbal regions of all animals at the earliest time point; specifically, the Foxp3-GFP$^+$ cells localized to the extravascular region of the limbal vessel arcade in the peripheral cornea (Fig. 1-4). Occasional Foxp3-GFP$^+$ cells were seen to transit through the circumferential vessels of the limbus but these were not enumerated. All Foxp3-GFP$^+$ cells observed in every examined tissue exhibited a similar small, round morphology, consistent with lymphocytes (Figure 1). No Foxp3-GFP$^+$ cells were observed elsewhere in the anterior segment, specifically in the iris, cornea, aqueous humor and ciliary body. Adult animals exhibited significantly greater numbers of Foxp3-GFP$^+$ cells in limbal regions than did young animals, with greater anatomical distribution, although they were still limited to the extravascular region of the limbal vessel arcade in the peripheral cornea (Figures 3 and 4). As a comparison, the presence of Foxp3-GFP$^+$ cells in the ear skin of naïve adult animals was also investigated, where they were readily noted to be evenly distributed in moderate numbers throughout the tissue (Figures 1-4). The earliest time point at which Foxp3-GFP$^+$ cells were visible in the skin and limbus of very young animals was also ascertained; no Foxp3-GFP$^+$ cells were noted in the eyes nor skin or 8 or 12 day old animals, but they were observed at 14

days of age, shortly after eye opening (12-13 days of age; Figure 5). Additionally, both bulbar (i.e., on the ocular surface) and palpebral (i.e., on the inner surface of the eyelid) conjunctivae of naïve adult animals were examined for the presence of Foxp3-GFP$^+$ cells; as these tissues can be imaged via noninvasive intravital microscopy only in a limited manner owing to their relative inaccessibility, freshly harvested tissue was also examined using the same intravital microscope. Whereas Foxp3-GFP$^+$ cells were sparsely present in bulbar conjunctivae, they were present at a relatively high density in palpebral conjunctivae (Figures 6 and 7).

Numerous effector T cells and Foxp3-GFP$^+$ cells infiltrate and are visible in the iris of uveitic eyes. With the goal of evaluating the role of regulatory T cells in the inflamed eye, we established an adoptive transfer model of murine uveitis wherein we could evaluate the presence in the iris of infiltrating, (red) CMTMR-stained ovalbumin-specific DO11.10 effector T cells and host (green) Foxp3-GFP$^+$ regulatory T cells. As mentioned previously, no host Foxp3-GFP$^+$ cells were present in the anterior segment of the eyes of recipient animals at baseline (i.e., prior to uveitis induction), aside from those localized to the extravascular region of the limbal vessel arcade in the peripheral cornea (Figures 1 and 2). After adoptive T cell transfer and intravitreal challenge with ovalbumin and 200 ng LPS, numerous red effector T cells and Foxp3-GFP$^+$ cells were visible in the iris stroma (Figures 8 and 9). In the contralateral eye, which concomitantly received an intravitreal injection of an equal amount of a control protein (bovine serum albumin, BSA, and 200 ng LPS), a small number of red effector T cells and host Foxp3-GFP$^+$ cells were visible in the iris stroma; these amounted to less than ten percent of those seen in the iris challenged with the specific antigen, ovalbumin, and were ignored in our analyses (Figures 8 and 10). The cell density in the ovalbumin-challenged eye peaked at 24 hours post injection (hpi) and remained elevated at 48 hpi before decreasing significantly by 72 hpi (Figure 9). Adoptively

transferred, antigen-specific effector T cells outnumbered host Foxp3-GFP$^+$ cells in each eye examined at the 24 and 48 hour time points by ratios of approximately 3:1 and 2:1, respectively, before being present in similar numbers at 72 hpi (Figure 11).

Absence of regulatory T cells during uveitis induction does not affect the onset, persistence nor overall numbers of iris-infiltrating effector T cells. For the purpose of evaluating the possibility that regulatory T cells influence the behavior of effector T cells in our uveitis model, we used recipient animals, BALB/c Foxp3-GFP-DTR, that allow the regulatory T cells to be transiently depleted, thus enabling the effect of regulatory T cell absence to be discerned. Prior to regulatory T cell ablation via intraperitoneal injection of DT (or PBS, in control animals), adult Foxp3-GFP-DTR recipient animals were examined via intravital epifluorescent microscopy for the presence of host Foxp3-GFP$^+$ cells in the anterior segment of the eyes at baseline. In contrast to what was observed in naïve Foxp3-GFP animals, where Foxp3-GFP$^+$ cells were visible in the extravascular region of the limbal vessel arcade in the peripheral cornea, no Foxp3-GFP$^+$ cells were visible in any examined ocular tissue, as well as conjunctiva and skin (data not shown). Subsequent to intraperitoneal injection of DT (or PBS), Foxp3-GFP-DTR recipient animals behaved and were treated identically to Foxp3-GFP recipient animals during the course of uveitis induction and evaluation. As with Foxp3-GFP recipient animals following adoptive T cell transfer and intravitreal challenge with ovalbumin, in non-Treg-depleted Foxp3-GFP-DTR recipient animals, numerous red effector T cells were visible in the iris stroma at 24 and 48 hpi before declining, whereas few were seen in BSA-challenged contralateral eyes (Figures 12 and 13). In Treg-depleted animals, red effector T cells were present in the iris stroma in comparable numbers as in Treg-replete animals at 24, 48 and 72 hpi (Figures 12 and 13). Unexpectedly, in non-Treg-depleted animals (i.e., PBS treated), several small, round Foxp3-GFP$^+$ cells were

faintly but reliably visible in the limbal regions of ovalbumin-challenged eyes at 24 hpi, admixed with numerous red effector T cells (Figure 14). These faint but reliably visible Foxp3-GFP$^+$ cells remained present at all observed time points in the antigen-challenged eyes of non-Treg-depleted animals, but were not visible in the eyes of Treg-ablated animals until 72-96 hours after intravitreal ovalbumin challenge (Figure 14). In addition to this *in vivo* assessment of Foxp3-GFP-DTR cell depletion, we assayed the DT-mediated ablation via flow cytometric analysis of the peripheral blood. Whereas no GFP$^+$ cells were observed in the samples from control animals, non-ablated Foxp3-GFP and Foxp3-GFP-DTR reporter animals exhibited significant numbers of GFP$^+$ cells, which were nearly absent from DT-treated animals, constituting an ablation frequency of greater than 98% (Figure 15). To confirm these findings, we crossed our Foxp3-GFP and Foxp3-GFP-DTR reporter animals and subjected the double-transgenic progeny to DT-mediated ablation, which showed similarly high efficiency (Figure 15). In order to further establish the absence of regulatory T cells in our DT-treated animals, we also examined our DO11.10, antigen-specific adoptive transfer inoculum for the presence of CD4$^+$CD25$^+$Foxp3$^+$ cells via flow cytometry. Our results demonstrate that these cells were present as only a low percentage of our *in vitro* culture, fewer than 2.5% of the CD4$^+$ lymphocytes (Figure 16), which constituted nearly 87% of the lymphocytes (Figure 17).

Characterization of the presence of iris-resident CD11c-YFP$^+$ cells and LysGFP$^+$ cells and their response to antigenic stimulus. It has been previously reported that tissue-resident CD11c$^+$ dendritic cells were observed by intravital microscopy to take up labeled antigen within tissues prior to migration to the draining lymph node (93, Melli 2009). In light of the experimental data suggesting that dendritic cells do not migrate from the intact eye, we chose to investigate this phenomenon in our non-invasive, ocular intravital microscopy system, using

CD11c-YFP and Lys-GFP reporter animals. Numerous CD11c-YFP$^+$ cells and LysGFP$^+$ cells were observed in the iris stroma of quiescent eyes. After intravitreal challenge with AF594 (red)-labeled protein, we observed that the CD11c-YFP$^+$ cells, in contrast to the LysGFP$^+$ cells and what has been reported previously for the iris (57, Becker 2003), did not appear to colocalize with the AF594$^+$ population when observed 24 hours later via intravital microscopy (Figures 19 and 21). In order to validate these findings, we observed the fixed iris tissue using a confocal microscope, wherein the differential uptake of labeled antigen by the two cell populations was confirmed (Figures 20 and 21). We took care to note the influx of numerous CD11c-YFP$^+$ cells into the intravitreally challenged eye, whereas the LysGFP$^+$ population remained stable after antigen challenge (Figure 21).

Characterization of the effect of regulatory T cells on the response of iris-infiltrating CD11c-YFP$^+$ cells to antigenic stimulus. The ability of regulatory T cells to control the infiltration of dendritic cells into an inflamed tissue has been reported previously in the pancreas, using a T cell-mediated, antigen-specific model and intravital imaging (94, Lee 2010). Given the unique immunologic characteristics of regulatory T cells and dendritic cells in the eye, we elected to investigate this behavior in our ocular intravital microscopy system, using CD11c-YFPxFoxp3-GFP-DTR reporter animals and the antigen-specific, DO11.10-mediated adoptive transfer uveitis model described above. As our read-out, we assessed the change in CD11c-YFP$^+$ cell density following adoptive transfer of DO11.10 effector cells and intravitreal challenge with the specific antigen (ovalbumin), in the presence or absence of recipient regulatory T cells. As a control, we imaged the untouched, contralateral eye prior to and 24 hours after intravitreal challenge with antigen, in order to evaluate for a sympathizing response of the iris-resident dendritic cells; naïve animals were imaged over the same time frame to establish a baseline.

CD11c-YFP^{+} cells were noted to exhibit significant changes in distribution in the iris in a sympathizing manner, whereas minimal day-to-day change was noted in the naïve animals (Figures 22 and 23). In our DO11.10 T effector adoptive transfer experiments using CD11c-YFPxFoxp3-GFP-DTR recipients, we noted no statistically significant difference between the CD11c-YFP^{+} cell response to antigenic stimulus in the presence or absence of regulatory T cells (Figure 24). The efficiency of Foxp3-GFP-DTR depletion was confirmed as stated previously, via flow cytometry and intravital assessment of the appearance of Foxp3-GFP^{+} cells.

Discussion

Uveitis is a common (200,000 persons/year in the U.S.; 95, Suhler 2008), oftentimes intractable disease whose clinical management, even with the most sophisticated immunomodulatory and immunosuppressive regimens, frequently leads to unsatisfying outcomes for the patient and the physician (96, Rosenbaum 2010). Regulatory T cells, whose advent in the last decade has been heralded due to their innate ability to control immune-mediated processes *in vivo*, represent a potentially novel approach to treating autoinflammatory diseases, including uveitis (97, Caspi 2011). In this study, we sought to undertake an initial characterization of the role of regulatory T cells in the healthy and inflamed eye using a murine model of T cell-mediated uveitis, so as to inform future attempts at employing regulatory T cells for therapeutic purposes in the setting of ocular inflammation (98, Allan 2008).

Our initial step was to determine what role, if any, exists for regulatory T cells in the naïve, healthy eye. Although it is well established that the intraocular environment promotes the conversion of infiltrating effector T cells into "induced" regulatory T cells, it is less clear if natural regulatory T cells contribute significantly to the immunosuppressive milieu of the naïve eye (99, Stein-Streilein 07). In humans, the greatest line of evidence casting doubt on a role for natural regulatory T cells in the maintenance of a quiescent intraocular environment comes from the disease IPEX (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked), wherein mutations in the transcription factor Foxp3 invariably result in multiorgan autoimmune disease owing to the lack of functional regulatory T cells (100, Wildin 2001). Although up to a dozen different organs have been described as being affected by autoimmune insults in this syndrome, the eye is not one of them (101, Wildin 2002). However, given the severity of the overall symptoms, which are fatal in the absence of bone marrow transplantation and systemic

immunosuppression, it is conceivable that ocular manifestations have been overlooked. This is especially true given the prevalence of blepharitis (eyelid inflammation) in IPEX patients, which could mask other ocular effects. Furthermore, as IPEX is characterized by neonatal (or even *in utero*) onset, with only rare cases of attenuated disease persisting into late childhood or adolescence, afflicted individuals are unable to articulate their symptoms, which therefore must be diagnosed by abnormal laboratory findings or direct physical examination. Indeed, although the eye has not been specifically described as being a target of autoimmune disease in IPEX, neither has a thorough ophthalmologic (i.e., slit-lamp) nor histologic examination of the eye proper been reported in the literature for these individuals. Undoubtedly, this is due to the impracticality of performing a fundoscopic examination on an uncooperative, ill infant, which would require general anesthesia and place the child at undue risk with dubious clinical benefit. This reality leaves open the possibility that the absence of natural regulatory T cells in humans in the setting of IPEX does in fact result in ocular inflammation, despite the lack of any direct description in the medical literature to support or refute this contention.

This line of reasoning, questioning whether or not natural regulatory T cells overtly suppress an inchoate autoimmune response against the eye proper, is buttressed by a strikingly parallel situation in mice as in humans: in scurfy animals, which mimic IPEX patients owing to mutations that deleteriously affect Foxp3 (previously known as scurfin) function, the absence of regulatory T cells results in a fatal, multiorgan autoimmune disease of neonatal onset that has not been reported to include the eye (102, Godfrey 1994). However, as stated above regarding the medical literature on IPEX patients, the evidence for such an effect with respect to the eye does not stand to scrutiny, owing to the lack of reports describing a thorough histologic examination of the eyes of scurfy animals. Furthermore, as seen in humans, blepharitis is a common

manifestation in scurfy mice, making even a cursory examination of the health of the eye in these animals a difficult task.

In light of this situation, wherein the role of natural regulatory T cells in contributing to the maintenance of immune homeostasis in the healthy mammalian eye is uncertain, we set out to address this question by first examining the healthy, naïve murine eye for the presence of regulatory T cells using non-invasive, intravital epifluorescent microscopy. To do so, we chose to utilize a regulatory T cell reporter animal, BALB/c Foxp3-GFP, that features several favorable characteristics. As with all BALB/c animals, these mice are albino, such that the eyes (and skin) contain no pigmentation that would render intravital imaging of these tissues, especially the iris, unfeasible. Additionally, as a BALB/c animal, they can serve as recipients for the adoptive transfer of congenic DO11.10 T cells, which have been used extensively in immunologic studies owing to their transgenic TCR that recognizes a peptide from ovalbumin (103, Murphy 1990); in this manner, we can investigate an antigen-mediated effector T cell event and the resultant host regulatory T cell response. This particular regulatory T cell reporter animal, formally known as C.Cg-*Foxp3*$^{tm2(EGFP)Tch}$, bears the advantageous distinction of having been cloned as a "knock-in" at the native Foxp3 locus in tandem with EGFP using a bicistronic molecular construct; this thoughtful design enables the independent translation of both proteins in a native form, allowing for Foxp3 and EGFP to perform their physiologic functions unperturbed by one another (104, Jeremiah 2011). As a consequence, not only are the animals healthy throughout their experimental lifespan (owing to proper Foxp3 activity), but the EGFP activity faithfully and strongly reflects Foxp3 activity (87, Haribhai 2007). In contrast to other regulatory T cell reporter animals that we have examined that either exist on the pigmented C57BL/6 background (105, Wan 2005; 106, Suffner 2010; 107, Fontenot 2005) and/or express the fluorescent reporter

molecule fused to another protein (108-9, Kim: 2007, 2009), this Foxp3-GFP animal harbors Foxp3-GFP$^+$ cells that are readily visible via intravital microscopy (Figure 1).

At first blush, our finding that Foxp3-GFP$^+$ cells are localized solely to a very discrete anatomical location in the anterior segment of the murine eye may come as a surprise. It has been believed that regulatory T cells, in their capacity as anti-inflammatory mediators, contribute to normal immune homeostasis via trafficking through peripheral tissues prior to migrating via lymph vessels to draining lymph nodes (110, Tomura 2010), whereby they maintain tolerance to self-antigens through interactions with lymph node-resident dendritic cells that had emigrated from the same tissue (111, Shevach 2009). However, this belief has been primarily based on observations from tissues that provide a barrier function, such as the skin and gut, wherein the immune system continuously confronts microbial and other foreign agents and where effector cells of the immune system are known to reside in large numbers (112, Matsushima 2010); in this setting, regulatory T cells play a vital role in promoting immune tolerance to innocuous antigens amidst ongoing immune responses to potential pathogens (113, Lan 2007; 114, Izcue 2008). From this perspective, it is not clear to what extent the previous observations would necessarily extrapolate to internal organs that are not exposed to the outside, microbial world, and that do not house armies of immune cells poised to engage them. This is especially true of the eye, which is well-known to use multiple organ-specific mechanisms to eliminate, subvert, or actively exclude effector immune cells, including lymphocytes, from its internal environment (45, Caspi 2006). In this context, our finding that Foxp3-GFP$^+$ cells are not visible in the cornea, iris, aqueous humor nor ciliary body, unlike in the skin, is entirely consistent with the notion that the healthy, uninflamed eye actively seeks to keep effector immune cells at bay. This reasoning is reinforced by the eye-specific phenomenon wherein eye-resident dendritic cells, unlike what

has been reported elsewhere in the body, have not been observed to migrate from the healthy eye to the draining lymph node, presumably to preclude the initiation in the periphery of an autoimmune response against sequestered ocular antigens (8, Dullforce 2004). In the absence of such homeostatic dendritic cell trafficking, it stands to reason that the necessity for a tandem migration of regulatory T cells to effect tolerance against ocular antigens in the periphery via interactions with dendritic cells in the draining lymph node is also obviated.

However, this explanation for the virtual absence of Foxp3-GFP$^+$ cells in the anterior segment of the murine eye raises a fundamental question about the function of regulatory T cells. In view of the underlying premise that regulatory T cell exert anti-inflammatory effects on their surroundings, an ostensibly equally plausible expectation regarding their potential presence in the eye would be that they actively contribute to the establishment and maintenance of the immunosuppressive intraocular environment by being plentiful, not absent. That is to say, regulatory T cells could theoretically offer protection against immunological insults in the intraocular setting through elaboration of anti-inflammatory molecules and/or direct action on effector cells, as is seen elsewhere in the body where commensal microbiota are not encountered (e.g., kidney, liver, salivary and lacrimal glands; 115, Ishamuru 2010), and yet seemingly inexplicably this potential strategy for boosting the anti-inflammatory arsenal in the eye is not employed. Recent reports would suggest, in fact, that the presence of regulatory T cells in the eye could present a risk to the maintenance of an anti-inflammatory environment because, despite long-held beliefs to the contrary, regulatory T cells have been newly shown to participate in and promote the immune response (116, Mellor 2011). Indeed, the very definition of a regulatory T cell has been drawn into question by experiments showing that CD4$^+$CD25$^+$Foxp3$^+$ cells can provide T cell "help" to a CD8$^+$ T cell-mediated anti-tumor response in a murine model

and in a viral infection model (117, Sharma 2010; 118, Lund 2008). The role of regulatory T cells in this setting has been described as one of "early responders," wherein they are rapidly attracted by chemokines to an inflamed tissue, and their arrival can dictate the course of the ensuing immune response by either promoting or inhibiting the activation of effector cells, such as $CD8^+$ T cells (116, Mellor 2011). Similarly, a recent publication by a separate group has demonstrated that regulatory T cells can assist the development *in vivo* of the inflammatory $CD4^+$ T cell subset, Th_{17}, by regulation of a critical cytokine in their differentiation, IL-2 (119, Chen 2011). Further experiments have drawn into question the stability of the regulatory T cell phenotype, suggesting that under certain inflammatory circumstances, "committed" $CD4^+CD25^+Foxp3^+$ cells can subsequently acquire effector T helper cell functions (120, Esposito 2010; 121, Sharma 2009); however, this claim remains unsettled, as other researchers have suggested that such purported regulatory T cell phenotypic "plasticity" is only a characteristic of a small population of uncommitted cells, whereas the greater population remains stable (122, Hori 2011; 123, Bailey-Bucktrout 2011; 124, Rubtsov 2011). This latter controversy notwithstanding, in light of this relatively newfound, paradigm-shifting knowledge regarding the potential role of regulatory T cells in promoting inflammation, it would appear possible that the near complete absence of Foxp3-GFP^+ cells from the eye that we observe serves to protect against this eventuality.

As stated previously, we did not observe the naïve murine eye to be completely devoid of Foxp3-GFP^+ cells, which were reproducibly visible via non-invasive, intravital microscopy in the extravascular region of the limbal vessel arcade in the peripheral corneas of all eyes that we examined (Figure 1). As the sole location of Foxp3-GFP^+ cells in the naïve eye that we observed, the role of the corneoscleral limbus in normal ocular physiology is of paramount interest.

Defined by the juncture of three distinct ocular tissues (cornea, iris and conjunctiva/sclera), the limbus presents an intriguing anatomical and physiological site in which regulatory T cells may reside. The presence of limbal vessels, be they of blood or putative lymphatic origin, in the vicinity of the Foxp3-GFP$^+$ cells does not appear to be a likely cause for their accumulation at this site; whereas the iris is highly vascularized and yet no Foxp3-GFP$^+$ cells were present (aside from the rare circulating cell), nor did we observe Foxp3-GFP$^+$ cells elsewhere in the peripheral cornea of young animals, where lymphatic vessels have recently been proposed to exist (125, Zhang 2011). A more likely and compelling explanation for the existence of numerous Foxp3-GFP$^+$ cells in close proximity to the limbal vessel arcade lies in the fact that limbal stem cells, which continuously repopulate the cornea and are essential to its survival, are widely believed to reside precisely at this same location in a unique microanatomical epithelial niche known as the palisades of Vogt (126, Goldberg 1982; 127, Hatch 2009; 128, Majo 2008; 129, Sun 2010). Limbal stem cells have been shown to exhibit intrinsic immunosuppressive properties, presumably as a mechanism to protect themselves and the tissue they repopulate from potentially deleterious immunologic insults (130, Holan 2010). Further evidence of the primacy of shielding stem cells from immune-mediated damage comes from a recent publication that demonstrated regulatory T cells providing a similar protective role to hematopoietic stem cells (HSCs) in an allograft model, whereby the allogeneic HSCs were protected against the host immune response via the activity of regulatory T cells in the bone marrow niche (131, Fujisaki 2011). Thus, it is not unprecedented that the Foxp3-GFP$^+$ cells that we observe in the naïve murine limbus may serve a similar function with respect to limbal stem cells. Adding to the potential significance of this purported association between regulatory T cells and limbal stem cells is the recognition that the limbus is the most common site for tumors of the ocular surface (132, Takács 2009); stem

cells have recently been appreciated to have a seminal role in the development of many solid tumors (133, Clevers 2011; 134, Rosen 2009), whereas the function of regulatory T cells in protecting solid tumors from the immune response is well-documented (135, Rouse 2007; 136, Munn 2011). In this context, the presence of Foxp3-GFP$^+$ cells in the young murine limbus that we observe suggests a potential dynamic for the putative association of regulatory T cells with limbal stem cells in the neoplastic process, one that would begin far earlier than has been appreciated through the study of conventional tumor models (137, Chen 2005).

This contention, that Foxp3-GFP$^+$ cells accumulate specifically in the extravascular region of the limbal vessel arcade of the peripheral cornea in order to confer greater immunoprotection to this tissue, is bolstered by the observation that adult animals harbor statistically significantly greater numbers of Foxp3-GFP$^+$ cells at this location compared to weanlings, whereas the rest of the eye remained devoid of these cells (Figure 3). In contrast, we did not observe an age-dependent difference between the numbers of regulatory T cells in the ear skin of weanlings versus adult animals (Figure 3). As for even younger animals, Foxp3-GFP$^+$ cells were first observed in the limbus at fourteen days of age, shortly after eye opening (12-13 days), which corresponds with the first observation of Foxp3-GFP$^+$ cells in the skin as well (Figure 5). This timeline of the initial observation of Foxp3-GFP$^+$ cells in the periphery is consistent with the onset of symptoms in scurfy animals, at approximately 14 days (138, Godfrey, 1991), whereas a direct comparison with the ontogeny of regulatory T cells described in humans, such as the initial presence of regulatory T cells in the fetal gut at 23 weeks of gestational age, is difficult to interpret (139, Weitkamp 2009). While inflation of regulatory T cell numbers with advanced age and antigen exposure has been described in the peripheral blood and in inflamed tissues (140, Zhao 2007; 141, Thomas 2007), this description of the early

temporal dynamics of Foxp3-GFP$^+$ cells in the periphery of mice in the absence of overt inflammation constitutes, to the best of our knowledge, the first account of this phenomenon in the literature. We find it noteworthy that Foxp3-GFP$^+$ cells arrive at two distinct tissues in the periphery simultaneously, despite the ongoing exposure of one, the skin, to microbial stimulus for a prolonged period of time beforehand whereas the other, the ocular surface, is newly exposed to antigen when Foxp3-GFP$^+$ cells arrive. We interpret this phenomenon as being consistent with the principle that the initial maturation of regulatory T cells and their eventual migration to non-lymphoid organs is not dependent on the foreign antigen exposure of the target organs, but rather on the self-antigens within the target organs, as has been suggested to explain the occurrence of scurfy-like symptoms in regulatory T cell-deprived germ-free animals (142, Chinen 2010). These observations regarding the delayed arrival of Foxp3-GFP$^+$ cells in peripheral organs would also appear to confirm the general belief, based on the manifestation of many of the symptoms associated with IPEX in the neonatal or even sterile *in utero* setting, that the primary role of regulatory T cells in suppressing an autoimmune response is exerted in the lymphoid tissue (143, Ochando 2005); this may be best exemplified by the report that regulatory T cells that are genetically incapable of expressing the homing receptors for the gut are nonetheless capable of exerting a profound anti-inflammatory effect in a colitis model via their activity in the mesenteric lymph nodes (144, Denning 2005). As for the specific persistence and accumulation of Foxp3-GFP$^+$ cells exclusively in the corneoscleral limbus in the adult murine eye, we believe this suggests a meaningful, rather than random, process, given their absence or scarcity elsewhere on the ocular surface (such as the cornea and bulbar conjunctiva, respectively), where presumably equal exposure to antigenic stimulus occurs.

In order to provide additional context to our investigations of the presence of regulatory T cells in the healthy eye proper, we also examined, in addition to the skin, the conjunctiva of the Foxp-GFP reporter animals; our observations of the presence of Foxp3-GFP$^+$ cells represent, to our knowledge, the first report of such cells in this tissue in healthy mice (145, Fukushima 2007; 146, Sumi 2009), with the only previous similar report being on rabbits (147, Nesburn 2007). Surprisingly, we also were not able to identify any description in the literature of regulatory T cells in the healthy conjunctiva of humans, despite the widespread clinical significance of inflammatory conjunctival disease (148, Niederkorn 2008). In contrast to the report on rabbits, which showed immunohistochemical images but unfortunately did not quantify the cell numbers, our findings show a dramatic difference in the cellular density of Foxp3-GFP$^+$ cells in the murine bulbar conjunctiva relative to those found in palpebral conjunctiva, as well as in ear skin (Figure 6). The finding of few Foxp3-GFP$^+$ cells in the adult murine bulbar conjunctiva relative to the skin is somewhat surprising, given that both tissues perform a barrier function and are confronted with continuous antigenic stimulus by the outside world. Especially perplexing is the disparity in the concentration of Foxp3-GFP$^+$ cells between the bulbar and palpebral conjunctivae, the latter of which contains a comparable number as in ear skin. Superficially, one explanation could derive from a gradient effect, whereby the bulbar conjunctiva is contiguous with the cornea, which contains no Foxp3-GFP$^+$ cells (with the notable exception of the limbus), whereas the palpebral conjunctiva is contiguous with the skin of the eyelid, which presumably contains Foxp3-GFP$^+$ cells in similarly high numbers as in the ear skin; it has been noted previously that comparable large numbers of regulatory T cells in the human body reside in the skin as circulate in the peripheral blood (149, Clark 2006; 150, Schaerli 2006), and even the majority of those circulating in the peripheral blood express a skin-homing receptor, known as cutaneous

lymphocyte-associated antigen (CLA; 151, Hirahara 2006), so it is conceivable that the neighboring skin may serve as a "reservoir" of regulatory T cells for the conjunctiva. We cannot, however, exclude the possibility that anatomical and/or functional differences of between the two tissues, which we are unaware, account for this disparity. From a teleological perspective, the presence of a high number of regulatory T cells in the palpebral conjunctiva may prove more beneficial to the ocular surface than it would be in the bulbar conjunctiva, given the direct apposition of the inner surface of the eyelid over the regulatory T cell-deficient cornea during eye closure; this is exemplified by the well-known salutary effect of the eyelid on the health of the ocular surface, including the common practice of suturing the eyelid closed (tarsoraphy) following corneal surgery (152, Lenčová 2011). The significance of this finding relative to human health is uncertain, given the apparent absence of information regarding the presence of regulatory T cells in the healthy human conjunctiva, as well as the suggestion that the rabbit conjunctiva, where such a dichotomy between the regulatory T cell concentrations in the bulbar and palpebral conjunctivae was not quantitatively demonstrated, is more anatomically similar than murine conjunctiva to that of humans (147, Nesburn 2007). Nonetheless, we interpret the non-uniform distribution of Foxp3-GFP$^+$ cells in the conjunctiva and in the corneoscleral limbus to be consistent with a functionally meaningful presence of regulatory T cells in these tissues; supporting this argument is the report of keratoconjunctivitis as one of the most prominent and common symptoms in the human autoimmune disease APECED (autoimmune polyendocrinopathy, candidiasis, ectodermal dystrophy, also known as APS-1 (autoimmune polyendocrinopathy (or polyglandular) syndrome type 1)), as well as in its murine counterpart, AIRE-deficiency (153, Chang 2006; 154, Yeh 2009). In this syndrome, defective expression of the AIRE (autoimmune regulatory) protein, a promiscuous transcription factor responsible for

promoting ectopic expression of tissue-specific proteins in the thymus, leads to a defect in central tolerance wherein self-reactive T cells fail to be deleted, resulting in multiorgan autoimmune disease (155, Fierabracci 2011). It has recently been recognized that, in additional to the principal failure in the induction of central tolerance in the APECED/AIRE-deficiency syndromes that can lead to self-reactive, T cell-mediated insults against the retina and ocular surface (156, DeVoss 2006; 157, DeVoss 2010), there is also a defect in regulatory T cell number and function (158, Ryan 2005; 159, Kekäläinen 2007; 160, Laakso 2010). Although the exact mechanistic nature of this regulatory T cell defect remains an object of active research (161, Pomié 2011), we take these various experimental and clinical reports to support our view of the functional significance of our findings of Foxp3-GFP$^+$ cells in the corneoscleral limbus and conjunctiva in terms of affording immunoprotection to these tissues; this interpretation is further supported by reports of limbal stem cell deficiency in APECED patients with severe corneal disease (162, Shah 2007; 163, Mohammadpour 2006), which our data suggest could be due to a local defect in regulatory T cell immunoprotective activity vis-à-vis the limbal stem cells.

After addressing the question of the potential role of regulatory T cells in the naïve murine eye by examining the presence of Foxp3-GFP$^+$ cells using intravital microscopy, we turned our attention to the potential role of regulatory T cells in the inflamed eye through investigation of an adoptive transfer uveitis model. In this set-up, we are able to visualize the presence of ovalbumin-specific, fluorescently-labeled DO11.10 cells (CMTMR, red) and host Foxp3-GFP$^+$ cells (green) in the murine iris, as with the naïve eye, using non-invasive, intravital microscopy. Although numerous animal models of uveitis exist (164, Agarwal 2004), this model has several advantages: in contrast to more popular murine models, our DO11.10-mediated

approach (which we term EOU, experimental ovalbumin-induced uveitis; 65, Rosenbaum 2008) produces a significant T cell infiltrate in the anterior segment of the eye, thus allowing direct visualization of the inflammatory process (56, Dullforce 2006; 165, Spencer 2008). In addition to permitting a non-invasive, and presumably physiologically unperturbed, view of effector cell activity in the anterior uveal tract (50, Becker 2002), this model more closely recapitulates the clinical setting wherein anterior uveitis predominates (166, Suhler 2003). In contrast, classical EAU (experimental autoimmune uveitis), which is induced via immunization of a susceptible animal with retinal antigens, results in T cell-mediated attack against the neural retina and nearby tissues in the posterior pole of the eye, precluding the use of intravital microscopy to observe this phenomenon on a cellular level (167, Horai 2010). Adoptive transfer EAU models, wherein animals expressing a transgenic protein in the lens (168, Lai 1999) or retina (169, Gregerson 1999) are infused with T cells targeting the transgene through immunization of donor animals, similarly develop uveitis that predominantly affects the posterior segment of the eye. While posterior uveitis does occur in humans, with significant detrimental effects for vision for those affected, it represents a minority of the cases that present to the ophthalmologist (170, Kaçmaz 2008). Another advantage of our adoptive transfer uveitis model is the ability to experimentally manipulate the donor cells as well as the recipient cells separately; thus, we can transfer fluorescently red-labeled, antigen-specific DO11.10 cells into a naïve animal whose regulatory T cells express the reporter protein GFP under the control of the Foxp3 promoter, and thereby study the dynamics of these two distinct cell populations in the response to intravitreal challenge with specific antigen (Figure 8). Furthermore, in the EOU model, the response to intraocular injection of specific antigen is swift and reproducible (Figure 9), with the contralateral eye serving as an internal control for the modest bystander inflammation resulting from the

inoculation of protein (Figure 10); this timeframe of disease onset compares favorably with classical EAU, wherein pathological onset takes weeks to develop (60, Tarrant 1998).

In our model, we see both antigen-specific donor effector DO11.10 T cells and host Foxp3-GFP$^+$ cells present in high numbers simultaneously in the specific antigen-challenged iris at twenty-four hours after injection, whereas very few cells of either type are visible in the contralateral, non-specific protein-challenged control eye; this antigen-specific accumulation is not unexpected for the DO11.10 effector T cells, which were restimulated *in vitro* for four days in the presence of their cognate ovalbumin-derived peptide after originating from an ovalbumin-immunized animal, but the presence of ostensibly specific-antigen unexposed Foxp3-GFP$^+$ cells at such an early time point suggests that they are attracted antigen-nonspecifically by the inflammatory milieu created by the effector DO11.10 T cells, in support of the recent findings suggesting an "early responder" role for regulatory T cells in the immune system (116, Mellor 2011). This interpretation is supported by the observation that the DO11.10 effector T cells and the Foxp3-GFP$^+$ cells exhibited a consistent ratio of approximately 3:1 and 2:1 at the twenty-four and forty-eight hour time points, respectively, per each eye examined (Figure 11), suggesting a direct role for the magnitude of the effector T cell presence, as manifested by inflammatory mediators, in determining the consequential amount of the regulatory T cell influx. This has been shown to be the case in various T cell-mediated murine models of inflammation, such as of the skin and CNS (EAE, experimental autoimmune encephalomyelitis), wherein antigen-specific T cells "condition inflamed sites for high-rate antigen-non-specific effector" T cell recruitment, specifically through the elaboration of the inflammatory molecules TNF-α and IFN-γ (171, Ghani 2009; 172, Lees 2008), and in a related model of uveitis, wherein DO11.10 T effector cells recruit neutrophils through the elaboration of IL-17 in a CXCR4-dependent manner (84,

Zhang 2009a; 173, Zhang 2009b); this phenomenon of secondary recruitment of antigen non-specific leukocytes to an inflamed site by antigen-specific T effectors has only recently been extended to include regulatory T cells in the setting of murine uveitis (174, Chen, 2006; 175, Kerr 2008). The reproducible ratio that we observe between the DO11.10 T effectors and Foxp3-GFP$^+$ cells in the inflamed iris likely reflects a combination of the relative expression levels by these two cell populations of chemokine receptors like CCR5, CCR6 and CCR7, which have already been implicated in T effector and/or regulatory T cell trafficking (176, Yurchenko 2006; 177, Reboldi 2009; 178, Yamazaki 2008; 115, Ishimaru 2010), and their relative responsiveness to inflammatory mediators present in uveitis, such as those mentioned previously, TNF-α and IFN-γ. These latter two have been strongly implicated in the pathogenesis of uveitis, with IFN-γ having been directly demonstrated to have a causal role in murine models of uveitis (EAU: 86, Luger 2008), while anti-TNF-α agents represent a mainstay of uveitis treatment in humans (179, Suhler, 2009). The effect of TNF-α on regulatory T cells is a matter of active research, as recent reports conflict about the role of this cytokine in supporting regulatory T cell function: while some have suggested that natural regulatory T cells and induced regulatory T cells are affected differently by TNF-α, owing to differential expression of TNF receptors, such that natural regulatory T cells would require signaling from TNF-α in order to exert their suppressive effect while it is dispensable for inducible regulatory T cell activity (180, Housley 2011; 181, Grinberg-Bleyer 2010), others suggest enhanced regulatory T cell suppressive effect in the absence of TNF-α signaling (182, Chee 2011). This debate aside, the disparity in the effect of TNF-α on natural and induced regulatory T cell populations may explain the clinical differences observed in the response of different uveitic entities to anti-TNF-α agents (183, Lim 2007), possibly reflecting varying roles of these two regulatory T cell populations in contributing to the

control of the inflammation at the tissue site versus in the lymph node in different disease states (184, Bilate 2010; 185, Zhang 2009c). Although the eye is well-known to convert many infiltrating T cells into induced regulatory T cells (186, Ke 2010), the infiltrating Foxp3-GFP$^+$ cells in our model are clearly fluorescent green as they accumulate in the iris vessels, prior to entering the eye proper, demonstrating that they are already expressing GFP and Foxp3; circulating regulatory T cells are believed to consist of natural and induced regulatory T cells in an approximately 2:1 ratio (187, Thornton 2010; 188, Verhagen 2010), which would give ample opportunities for both cell populations to infiltrate the eye from the peripheral blood and prevent us from determining their relative proportions in our uveitis model using our current approach. As for the persistence of the effector DO11.10 and Foxp3-GFP$^+$ T cells in our model, we observe a high concentration of cells for 48 hours after intravitreal challenge before the cell density dissipates. Compared to other models of uveitis targeting native antigen where the cellular infiltrate can persist for many days or even weeks (189, Mattapallil 2011), the observed cellular density in our model persists for a brief amount of time; we don't believe that this limited duration of inflammation reflects the clearance of injected antigen through normal ocular outflow pathways, as we can observe intravitreally injected fluorescently-tagged protein to persist in the eye for over a week in phagocytic, iris-resident cells (65, Rosenbaum 2008). Evidence from other uveitis models would suggest that A/RICD (activation/restimulation-induced cell death; 190, Snow 2010) contributes to the elimination of the effector DO11.10 T cells in the uveitic eye, with Th17 cells being more resistant and persisting longer than Th1 cells (191, Shi 2009); this process has been attributed to the high levels of FasL in the eye (192, Ferguson 2007). As regulatory T cells have been shown to be resistant to A/RICD (193, Weiss 2011), their concomitant disappearance, along with effector DO11.10 T cells, by three days after entry in the

inflamed eye in our uveitis model presumably is not attributable to A/RICD, and suggests that it is the absence of the effector T cells and the corresponding resolution of the inflammation that is responsible for regulatory T cells no longer being actively maintained at the site. This contrasts with the persistence of Foxp3-GFP$^+$ T cells that we observe in the naïve murine limbus, and reinforces the dichotomy between the intraocular environment, where these cells appear to be actively excluded, and the limbus, where they specifically accumulate.

The presence of Foxp3-GFP$^+$ cells in the uveitic iris that we observe begs the question of what contribution, if any, these cells are making to the resolution of the inflammation. It is well-known that the eye possesses many intrinsic mechanisms for avoiding, minimizing and terminating inflammation in its intraocular environment (194, Horie 2010; 195, Mochizuki 2010), as discussed previously, and the role of regulatory T cells in participating in the control of uveitis has been investigated by many researchers, although rarely while addressing the question of the site of action of the anti-inflammatory effect. It was first observed that three-day-old thymectomized animals, whose thymus-resident regulatory T cells were eliminated while their effector T cells were allowed to egress to the periphery prior to day three, would spontaneously develop uveoretinitis and other organ-specific autoimmune disease if continuously exposed to anti-CD25 antibody (196, Taguchi 1996), which preferentially depletes regulatory T cells. Although this stands in contrast to experiments with scurfy animals lacking regulatory T cells, where ocular disease has not been described, this apparent discrepancy likely reflects the fact that the investigators specifically examined the eye (197, Takeuchi 2004). Attributing the site of action of the immune defect in this model to central or peripheral tolerance (i.e., acting within or outside the thymus), however, is complicated by the later finding that mice harbor a second thymus in their neck (198, Terszowski 2006; 199, Miller 2006). While central tolerance

mechanisms relating to thymic expression of retinal antigens have continued to be evoked to explain the variable susceptibility of different mouse strains to uveitis following immunization (200, Avichezer 2003), it has become clear that natural (i.e., thymus-derived) regulatory T cells contribute to the protection from retinal antigen-induced uveitis, regardless of their antigen-specificity, as their elimination through anti-CD25 administration heightened EAU pathological scores (201, Grajewski 2006). Other investigators, using a neoantigen virally transduced in the retina, showed that intravenous administration of *in vitro* activated, but not naïve, antigen-specific $CD4^+CD25^+$ "regulatory" T cells could reduce antigen-specific T cell infiltration of the eye (202, Terrada 2006). Subsequent authors demonstrated that adoptive transfer of natural, unstimulated regulatory T cells of undetermined specificity could ameliorate EAU against a native antigen by inhibiting effector T cell proliferation in the spleen, but not draining lymph node (203, Keino 2007), whereas others reported on the EAU-protective effect conferred by generating induced regulatory T cells of retinal antigen-specificity through ectopic (liver) expression of the ocular protein that was partially reversed by anti-CD25 treatment (204, Silver 2007). Induced regulatory T cells generated within the eye have also been implicated in the reduction of EAU pathology (205, Sugita 2009). These findings of the beneficial effect of regulatory T cells on the course of uveitis have been extended to rat models of EAU, wherein regulatory T cells from the eye of an animal recovering from monophasic uveitis could decrease the symptoms of animals experiencing recurrent uveitis (206, Ke 2008); this is the first study to show that eye-derived regulatory T cells have a suppressive effect in uveitis. In a separate rat model of ocular inflammation that has the distinction of producing anterior uveitis (EAAU), the tolerizing effect afforded by regulatory T cell after intravenous exposure to antigen in uveitis-susceptible animals has been attributed solely to those residing in the lymph nodes (207, Matta

2008), although eye-resident cells were not examined (208, Matta 2010). Further focus on the lymph node as the site of action of EAU-suppressive activity came from studies using retinal peptide-pulsed, tolerogenic DCs that conferred EAU protection through activation of regulatory T cells in lymph nodes, although the eye was not examined for their presence (209, Lau 2008). These observations were extended in another study wherein the dynamics of regulatory T cell numbers in draining lymph nodes was examined in correlation with the course of EAU; regulatory T cells were observed to expand and contract in synchrony with the severity of symptoms, although the eyes were not examined for their presence (210, Sun 2010). Mechanistically, it has recently been shown that the absence of IL-6, a crucial cytokine in the differentiation of inflammatory Th17 cells (211, Korn 2008), suppresses EAU and promotes regulatory T cell activity in the lymph node; the eyes were not examined for their presence (212, Haruta 2011). Given the plethora of experimental evidence strongly supporting a role for regulatory T cells in controlling uveitis in various experimental models, many groups have investigated different modalities for stimulating their activity in this setting: successful interventions have been reported to include anti-CD3 (TCR) antibody (213, Ke 2011); intravenously administered DNA encoding a retinal antigen (204, Silver 2007); Galectin-1, an endogenous lectin (214, Toscano 2006); anti-LFA-1a (adhesion molecule) antibody (215, Ke 2007); inhibitory peptide analogs of a major retinal antigen (216, Cortes 2008); an amino acid copolymer (217, Yin 2009).

Despite this multitude of intensive investigations into the role and potential therapeutic benefits of regulatory T cells in the setting of uveitis, no study has clearly analyzed the presence and function of regulatory T cells in the inflamed eye itself, in particular with regard to their local effect on infiltrating, inflammatory T cells; instead, as illustrated in the preceding

discussion of EAU articles, the primary interest has been on the activity of regulatory T cells in lymphoid tissue, especially their interactions therein with dendritic cells. In light of the previously mentioned particularity of ocular immune privilege wherein dendritic cells and non-soluble antigen have not been demonstrated to migrate from the healthy eye to the draining lymph node (46, Camelo 2006), this near-exclusive focus on regulatory T cell activity in the lymph node is of questionable physiologic validity. This perspective is reinforced by the recent recognition that regulatory T cells are present in target tissues (218, Sather 2007) and can contribute to or inhibit a nascent immune response (116, Mellor 2011). For these reasons, we decided to examine the role of regulatory T cells in our experimental ovalbumin-induced uveitis model, using Foxp3-GFP-DTR animals (BALB/c) as the recipients of DO11.10 (BALB/c) T effector cells, through non-invasive, intravital microscopy. Foxp3-GFP-DTR mice, also known as DEREG (DEpletion of REGulatory T cells), have been used extensively to characterize the function of regulatory T cells in numerous immunologic processes, owing to the reliable and exclusive depletion of Foxp3-DTR^{+} regulatory T cells in these animals following administration of diphtheria toxin (88, Lahl 2011). It should be noted that while diphtheria toxin-mediated ablation of cells expressing the diphtheria toxin receptor (DTR) under the control of a cell type-specific promoter is a well-established technique (219, Saito 2001), the use of this particular approach with the Foxp3 promoter has drawn criticism, owing to articles by a single group suggesting that Foxp3 expression is not limited to regulatory T cells but instead is expressed widely in epithelial tissue (220, Zuo 2007; 221, Liu 2009). This group has contended that the immunopathology described in Foxp3-DTR animals following diphtheria toxin administration results not from a scurfy-like disease owing to absence of suppression of self-reactive T cells by the depleted Foxp3-DTR^{+} regulatory T cells (222, Kim 2007), but rather from the widespread

ablation of Foxp3-expressing epithelial cells in numerous tissues throughout the animal (223, Chen 2008). This contention defies the prevailing and well-established paradigm that Foxp3 expression in mice is specific to regulatory T cells (107, Fontenot 2005), and has been directly challenged and refuted by a thorough investigation that has not reproduced its findings (109, Kim 2009). Much more recently, a second group has reported expression of Foxp3 in a very discrete, minor subpopulation of macrophages ("Foxp3$^+$ macrophages") that they report to exhibit anti-inflammatory properties much akin to regulatory T cells (224, Zorro Manrique 2011). Although these results have not been independently confirmed, the expression of Foxp3 in a discrete cell population of hematopoietic origin and anti-inflammatory function does not, in contrast to the publications by the group of Liu et al. (221, 2009), directly contradict the wealth of data related to Foxp3-specificity and ablation in regulatory T cells (225, Leavy 2011), nor is it inconsistent with our findings. It should be noted with due caution, however, that the Zorro Manrique manuscript prominently cites the findings of Zuo et al. (220, 2007) and Chen et al. (223, 2008) without acknowledging their refutation by Kim et al. (109, 2009). As for our studies of adoptive-transfer uveitis in Foxp3-DTR recipient animals, we elected to expose the animals to diphtheria toxin prior to the adoptive transfer and antigen challenge, such that we could assess the effect of ablation of Foxp3-DTR$^+$ T cells on the onset and persistence of effector T cell infiltration in our model; as this event occurs soon after intravitreal injection of specific antigen, reaching a peak by twenty-four hours and remaining at a high level for only a few days before disappearing, the reported five-day window of Foxp3-DTR$^+$ ablation would allow us to examine the entire inflammatory course in our model in the absence of the Foxp3-DTR$^+$ cells. An advantage of this technical approach of adoptive effector T cell transfer over others, wherein the DT animal is also actively immunized, has been demonstrated in a recent report wherein

concomitant DT-challenge and active immunization is lethal for the animals (226, Meyer Zu Hörste, 2010). Alternatively, in models such as EAU where the onset of symptoms takes many days or even weeks to develop, the DT-challenge can be timed so as not to overlap with the active immunization, but this eliminates the possibility of examining the effect of Foxp3-DTR^{+} T cell absence on the entire course of the immunologic process in a given animal. In our EOU model, the dual phenomena of absence of homeostatic dendritic cell migration from the eye to the draining lymph node and the early onset of iris infiltration by *in vitro*-activated, antigen-specific DO11.10 effector T cells allows us to study the effect of Foxp3-DTR^{+} cell ablation on the local behavior of iris-resident effector cells in relative isolation from distal control by events in secondary lymphoid organs. In control and experimental animals, we observed antigen-specific DO11.10 effector T cells to be present in comparable numbers at all observed time points, suggesting that systemic and local absence of Foxp3-DTR^{+} cells does not influence the course of effector T cell infiltration and eventual clearance in our uveitis model. This result is open to multiple interpretations: a first, and most probable, interpretation is that, in contrast to what is seen in many EAU models, the absence of regulatory T cells in the recipient animal and especially in the eye itself has no bearing on the behavior of transferred effector T cells in our particular uveitis model. While there may be many explanations for this outcome, it is not without precedent as other T cell-mediated models of autoimmune disease have shown similar findings, such as in a CD8^{+} T cell-mediated model of autoimmune lung disease, wherein CD4+CD25+Foxp3^{+} regulatory T cells were shown to be dispensable for controlling the disease (227, Tosiek 2011). In previous EAU experiments wherein the effect on intraocular pathology of conditional depletion (via anti-CD25 administration) of regulatory T cells has been examined, the affected animal was immunized systemically against retinal antigens, and the disease

symptoms took weeks to develop through a mechanism shown to require activation of antigen-specific effector T cells in the draining lymph node (e.g., 201, Grajewski 2006; 204, Silver 2007; Sun, 2010; 212, Haruta 2011); in this setting, it is clear that the regulatory T cells have an opportunity to intervene in and disrupt a nascent immune response through interactions with lymph node-resident dendritic cells, a crucial step which is largely bypassed in our model. In the rare setting of adoptive transfer experiments of retinal antigen-specific effector T cells in a regulatory T cell-depleted animal (194, Takeuchi, 2004), the onset of disease remains significantly delayed compared to our model (i.e., four weeks vs. twenty-four hours), with demonstrated expansion of effector and regulatory T cells in the secondary lymphoid tissue, suggesting that this site remains crucial for controlling the development of the immune response in this experimental approach; a corollary to this line of reasoning is that the activation of effector T cells in the lymph node that has been used frequently to evaluate the effect of regulatory T cell depletion on EAU in other models does not necessarily equate with intraocular effector T cell-mediated events, as has been shown in a T cell-mediated lung inflammation model wherein the T cell response in the draining lymph node and target organ following systemic regulatory T cell ablation were dissimilar (227, Tosiek 2011). To date, our current results represent the first account of the effect of regulatory T cells on longitudinal effector cell dynamics in the uveitic eye, particularly in the absence of prevailing immunoregulatory control by the regional lymph node. In the context of a fairly moderate and short-lived effector cell infiltration into the intraocular environment relative to the prolonged and severe pathology observed in EAU, our findings suggest that the eye is fully capable of controlling a moderate inflammatory insult using its innate immunosuppressive mechanisms, and that a regulatory T cell-mediated mechanism of controlling an ongoing inflammatory response is necessary only in

the course of durable, extensive tissue damage. The persistence of specific antigen may be one fundamental component in this difference, as although we observe labeled antigen for at least a week after intravitreal injection in iris-resident phagocytic cells (50, Becker 2002), its availability for eventual processing and presentation by dendritic cells in the draining lymph node is likely negligible compared to that of native ocular antigens, which importantly remain present in the target tissue for ensuing recognition by activated effector T cells. Furthermore, this apparent lack of a perceivable effect of regulatory T cell absence on effector T cell dynamics in our uveitis model suggests that the numerous Foxp3-GFP^{+} cells that are present in the irides of the uveitic Foxp3-GFP reporter animals at twenty-four hours after intravitreal antigen-challenge are not exerting an appreciable local anti-inflammatory effect, such as through direct cell-to-cell contact with effector T cells or iris-resident DCs, nor through elaboration of meaningful amounts anti-inflammatory mediators like TGFβ (that are already present in high concentrations; 14, Taylor 1999); as a counterpoint, it is possible that such an effect does occur but is below our detection threshold, although the infiltrating cell numbers in the regulatory T cell-depleted animals do trend to lower numbers, but not in a statistically significant manner (Figure 13), whereas one would expect an increase if a functionally important suppressive effect were relieved. It is notable that, given the recent evidence of regulatory T cells as being capable of promoting T cell-mediated incipient immune responses (117, Sharma 2010), the absence of regulatory T cells during uveitis induction in our model did not lead to a delay of onset nor affect the overall numbers of infiltrating effector T cell; this result would have been expected if regulatory T cells, which are observed to invade the newly uveitic eye in large numbers at an early time point in Foxp3-GFP^{+} animals, had a role in promoting the immune response in this setting. As the local environment is believed to be crucial in determining the physiologic role

assumed by regulatory T cells (116, Mellor 2011), our interpretation is that the intraocular immunosuppressive environment, such as the abundance of newly activated TGFβ in the aqueous humor of the uveitic eye (228, Taylor 2009), holds sway in the setting of moderate inflammation seen in our model, preventing infiltrating regulatory T cells from adopting an inflammatory phenotype. From a technical perspective, we confirmed the ablation efficacy of the Foxp3-GFP-DTR^{+} cells in our initial experiments through a conventional approach, by assaying the peripheral blood of the experimental and control animals for GFP-positivity in lymphocytes on day three via flow cytomety, after challenge with diphtheria toxin on days one and two; we obtained a depletion rate of approximately 99% (Figure 15). However, fortuitously, we observed that the control, PBS-injected animals all had faint but unmistakably GFP^{+} cells that were clearly visible in the limbus of the antigen-challenged eyes when examined via intravital microscopy, where fluorescent red-stained DO11.10 cells also accumulated in high numbers. These cells, which were small and round, consistent with lymphocytes, were absent from the limbi of the antigen-challenged eyes of diphtheria toxin-injected animals for the first two days after intravitreal challenge with specific antigen, appearing at the end of the third day (Figure 14). This observation correlates with the published report of the depletion of Foxp3-GFP-DTR^{+} cells lasting only a brief time, five days after DT challenge (88, Lahl 2011), before the cells rebound and demonstrates that the evaluation of the ablation efficacy of the Foxp3-GFP-DTR^{+} cells via flow cytometry corresponded directly with the absence of these cells via intravital microscopy in the target organ. We suspect that we did not observe Foxp3-GFP^{+} cells in the naïve limbus of Foxp3-GFP-DTR^{+} animals via intravital microscopy, as was the case with the Foxp3-GFP^{+} animals (Figure 1), because unlike in the former animal the two transgenic proteins, GFP and DTR, were expressed as a fusion protein, with DTR being an integral membrane protein, which

has been observed previously to decrease significantly the visibility of the fluorescent reporter protein (229, Jung 2002); the subsequent observation of Foxp3-GFP$^+$ cells in the limbi of the Foxp3-GFP-DTR animals after intravitreal antigen challenge likely reflects the high number of cells invading the limbus in the inflamed eye, allowing for a greater likelihood of observing them, as well as the greater optics provided by the dark background of the limbal vessels in contrast to the light green background of the iris stroma when viewing peripheral cornea and iris. Given the reproducible correlation of the *in vitro* and *in vivo* assessments of Foxp3-GFP-DTR ablation, as well as our primary interest in evaluating the effect of Foxp3-GFP-DTR absence in the eye itself, and for technical ease, we elected to evaluate the efficacy of Foxp3-GFP-DTR depletion in all of our challenged animals via "*in vivo* flow cytometry" (230, Fan 2010); in none of our experimental animals were Foxp3-GFP$^+$ cells observed prior to day five after onset of ablation. For this reason, we do not believe that our primary finding of no effect of Foxp3-GFP-DTR cell absence on effector T cell dynamics in our uveitis model can be attributed to an incomplete ablation of these cells and the incomplete absence of regulatory T cell-mediated suppression that would result. Examination of double-transgenic Foxp3-GFPxFoxp-GFP-DTR animals after diphtheria toxin administration revealed a lesser, although still elevated, efficacy of Foxp3-GFP$^+$ cell depletion in the peripheral blood than in the single-transgenic animals (Figure 15: approximately 90% vs 99%). To further validate our findings regarding the effect of regulatory T cell absence on effector T cell behavior in our uveitis model, we examined the adoptive transfer inocula containing *in vitro* activated DO11.10 cells from a whole splenocyte culture for the presence of CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cell contaminants that would be antigen-specific and immune to diphtheria toxin-mediated ablation. It has been shown previously that greater than 95% of the adoptively-transferred cells in our model are DO11.10 T cells (56,

Dullforce 2006); we found a very small fraction, less than 2.5% of the $CD4^+$ lymphocytes, to be $CD4^+CD25^+Foxp3^+$ cells (Figure 16), corresponding with at least five-fold fewer than what has been reported to be required for suppression in EAU models (203, Keino 2007). In summary, we find that in our experimental ovalbumin-induced uveitis model, the dynamics of DO11.10 effector T cell infiltration of the specific antigen-challenged eye is not affected in terms of onset, persistence, nor overall numbers in the absence of endogenous regulatory T cells, suggesting that the companion $Foxp3^+$ regulatory T cells observed at early time points in this target tissue during the inflammatory process do not substantively hinder nor promote effector T cell behavior in the setting of moderate intraocular inflammation.

In a separate line of experiments, we sought to further investigate the potential role of regulatory T cells in the ocular response to inflammatory insult by examining the behavior of dendritic cells in our uveitis model. Dendritic cells have been shown to respond to inflammatory activity in other settings in a manner controlled by regulatory T cells, although given the particularity of dendritic cell biology in the eye, it is unclear to what degree this observation extends to uveitis (94, Lee 2010). In this publication, intravital imaging of the pancreas in a mouse model of T cell-mediated autoimmune diabetes was used to demonstrate for the first time that dendritic cells from the periphery invade the inflammatory lesions in a manner controlled by natural regulatory T cells. Reporter animals expressing enhanced yellow fluorescent protein (YFP) under the control of the dendritic cell-specific promoter CD11c (89, Lindquist 2004) were used to visualize the dendritic cell infiltration of the inflamed pancreatic islets in a technique that is readily amenable to analysis in our system of intravital ocular imaging. Using our adoptive transfer approach with antigen-specific DO11.10 T cells providing the inciting inflammatory insult, we set out to image the arrival of dendritic cells from the periphery in Foxp3-GFP-

DTRxCD11c-YFP animals in the presence and absence of systemic regulatory T cells. However, before doing so, we were determined to establish the baseline presence and activity of CD11c-YFP$^+$ dendritic cells in the healthy, naïve murine eye before examining this behavior in uveitis. In order to achieve this, we chose to characterize the response of iris-resident CD11c-YFP$^+$ dendritic cells to exposure to foreign protein, given the central role of intravitreal injection of ovalbumin to the initiation of our uveitis model. Previous experiments in mice using intravital microscopy but different labeling techniques suggested that the tissue-resident dendritic cells would take up protein in a both a homeostatic and inflamed setting, but neither commented on the infiltration of circulating dendritic cells in response to inflammation or protein challenge: the first publication examined the murine iris using a novel *in vivo* immunohistologic approach based on the local injection of differentially labeled anti-CD11c antibody and protein (57, Becker 2003), whereas the second paper used the same CD11c-YFP reporter animals as in our experiments to visualize the uptake by pancreatic islet-resident CD11c-YFP$^+$ dendritic cells of endogenous EGFP expressed transgenically using a pancreatic islet-specific promoter (231, Melli 2010). Unexpectedly, after examination of the iris of CD11c-YFP reporter animals twenty-four hours after intravitreal injection with fluorescently-conjugated protein (AF594), we observed that our results contrasted with both publications; whereas both publications showed uptake of fluorescent protein by CD11c$^+$ dendritic cells, our intravital analysis revealed no discernible uptake by the CD11c-YFP$^+$ dendritic cells, but rather by a second, uniformly phagocytic cell population (Figure 19). To establish the identity of the phagocytic cell population taking up antigen in the murine iris, we utilized a second transgenic reporter animal, Lys-GFP (BALB/c), that expresses EGFP under the control of the lysozyme M promoter, resulting in green fluorescent macrophages and neutrophils (90, Faust 2000). Examination of the

irides of Lys-GFP animals via intravital microscopy twenty-four hours after intravitreal challenge with fluorescently labeled protein (AF594) revealed that, in contrast to the experiments with the CD11c-YFP animals, the vast majority of the Lys-GFP cells were double-labeled, with no apparent $AF594^{+}$ cells (Figure 19). In order to confirm these findings, we examined the fixed, dissected irides of both CD11c-YFP and Lys-GFP animals twenty-four hours after intravitreal challenge with fluorescently conjugated protein *ex vivo*, using confocal microscopy; using this approach, we were able to generate three-dimensional images of the different fluorescent cell populations (Figure 20), and establish that whereas virtually no CD11c-YFP^{+} dendritic cells showed overlap with AF594 fluorescence, the vast majority of the Lys-GFP^{+} phagocytic cells did (Figure 21), consistent with the findings of a study on iris-resident phagocytic cells of rats using different cell markers (232, Camelo 2003). The disparity of these findings with the prior report on iris-resident CD11c dendritic cells by Becker et al. (57, 2003) can likely be attributed to different labeling techniques, as *in vivo* labeling of CD11c cells via injection of fluorescently conjugated antibody is liable to erroneous labeling via non-specific antibody binding, which may have occurred despite the use of isotype antibody controls; indeed, close examination of the labeling pattern in that publication reveals a striking similarity between the distribution of the cells labeled with the anti-CD11c antibody and the Lys-GFP cells that we observe via intravital microscopy. As for the second paper, wherein the same CD11c-YFP reporter animal is used and examined via intravital microscopy, the apparent disparity in uptake of fluorescent protein by CD11c-YFP^{+} dendritic cells cannot easily be attributed to technical differences; in this case, it would appear that tissue-specific functional differences in dendritic cells, as is already well-known to be the case for these cells in the eye regarding various immunologic properties relative to those residing elsewhere in the body (19, Camelo 2005), may be responsible. Alternatively, it

is possible that our technical approach, specifically the intravitreal injection of foreign antigen into the eye, engenders a non-physiologic, inflammatory state in the intraocular environment that changes the behavior of the iris-resident dendritic cells from their baseline condition, and that what we observe is an adaptive response. This hypothesis is supported by the observation that in the Lys-GFP animals, bright fluorescent neutrophils were seen to infiltrate the entire iris at early time points, precluding examination of the pattern of protein uptake in these animals until the twenty-four hour point, at which juncture they had largely disappeared and were only persistent in the iris vessels (Figure 20). Indeed, similar results regarding a transient, mild inflammatory infiltrate following intravitreal injection of non-specific protein have been reported previously (56, Dullforce, 2006). However, even in this hypothetical scenario, our results are not consistent with those of either article regarding the uptake of antigen by tissue-resident dendritic cells, as both reports describe the dendritic cells as taking up labeled antigen during inflammatory states, unlike what we observe. With regard to Becker et al. (57, 2003), we used nearly the same technical approach as they did to expose the iris-resident cells to fluorescently-labeled protein, so the physiologic state of the iris-resident dendritic cells, be it homeostatic or adaptive in nature, is almost certainly identical; whereas they introduced the labeled protein through an anterior chamber injection, we did so through an injection into the vitreous cavity. Owing to the physiologic flow of intraocular fluid anteriorly from the vitreous cavity through the pupil and into the anterior chamber (233, Acott 2008), it is difficult to imagine a biologically significant difference between the two routes of antigen injection. Moreover, whereas anterior chamber injections are notoriously implicated in inducing systemic tolerance to the foreign antigen through the complex immune response termed anterior chamber-associated immune deviation (ACAID; 234, Zhang-Hoover 2007), a virtually identical phenomenon has been described for

antigen injected into the vitreous cavity (VCAID; 235, Sonoda 2005), suggesting that protein introduced through either route results in the same physiologic result in the eye. With regards to Melli et al. (231, 2010), in addition to the uptake of endogenous fluorescent protein by pancreas-resident CD11c-YFP^{+} dendritic cells that they describe under homeostatic conditions, they report increased fluorescent antigen uptake by these same cells under T cell-mediated inflammatory conditions as witnessed via intravital microscopy, providing an even starker contrast with our observation of virtually no double-labeling of these same cells after exposure to labeled protein in the eye.

Having established the novel finding that iris-resident CD11c-YFP^{+} dendritic cells do not appear to take up antigen under relatively homeostatic or potentially inflammatory conditions, we turned our attention to the potential influence of regulatory T cells on the trafficking of dendritic cells to the uveitic eye, as described to be the case in the inflamed pancreas by Lee et al. (94, 2010). While the dynamic behavior of certain subsets of myeloid-derived cells in the eye has been examined, such as the turnover of Cx3cr1^{+} macrophages in the uveal tract (236, Kezic 2008), very little is known about the trafficking of CD11c $^{+}$ dendritic cells to the eye under homeostatic or inflammatory conditions (237, Forrester 2010). In order to further our understanding of this phenomenon, we first examined CD11c $^{+}$ dendritic cells in the naïve eye to establish a baseline before focusing on the response to an intraocular inflammatory event. Using non-invasive, intravital microscopy, we observed that CD11c-YFP^{+} dendritic cells exhibit relatively little visible day-to-day dynamic behavior in the naïve iris (Figure 22), in contrast to the reported active migration of CD11c^{+} dendritic cells in other organs, such as the skin (238, Henri 2010). Following intravitreal injection with antigen, numerous newly arrived CD11c-YFP^{+} dendritic cells from the vasculature were observed to populate the iris, with the density of

CD11c-YFP$^+$ dendritic cells significantly increased at the twenty-four hour time point (Figure 21); by comparison, no change was observed in the overall number of iris-resident phagocytic fluorescent cells in Lys-GFP$^+$ animals. These results confirmed that the general phenomenon reported by Lee et al. (94, 2010) regarding the infiltration of CD11c-YFP$^+$ dendritic cells to a site of inflammation was equally valid for the immune-privileged eye as for their model organ, the pancreas, despite the known peculiarity of ocular dendritic cell physiology (239, Camelo 2004). With the intention of using the contralateral eye as an uninjected control for the infiltration of CD11c-YFP$^+$ dendritic cells to a specific antigen-driven, T cell-mediated inflammatory event in the opposing eye, we investigated the potential for a "sympathizing" effect on the uninjected, contralateral eye, as has been described to occur in the retina in an unmanipulated eye following injury to the contralateral eye (240, Lehmann 2010). Subsequent to the intravitreal injection of antigen, we noticed a significant increase from baseline of the dynamic behavior of the iris-resident CD11c-YFP$^+$ dendritic cells, with approximately forty percent of the observed cells present in a location different from what had been observed twenty-four hours earlier (Figure 23). This novel "sympathizing" effect in the iris is likely due to the bystander crossover effect of neuronal projections from the damaged "exciting" eye (241, Macharadze 2009), which produce neuropeptides capable of influencing DC activity (242, Nijhuis 2010); in terms of affecting our experimental approach, the overall "sympathizing" effect was modest and negligible relative to the numbers of CD11c-YFP$^+$ dendritic cells invading the antigen-challenged eye (Figure 21). Having established the background behavior of CD11c-YFP$^+$ dendritic cells in the naïve eye and in response to antigen-specific inflammatory insult, we sought to determine what influence, if any, regulatory T cells may exert on this phenomenon, as has been demonstrated to be the case in the T cell-mediation inflammation in the pancreas (94, Lee 2010). Using CD11c-YFPxFoxp3-

GFP-DTR animals, we were able to observe the infiltration of CD11c-YFP$^+$ dendritic cells into the murine iris in our DO11.10 adoptive transfer uveitis model via intravital microscopy in the presence or absence of regulatory T cells, following challenge with PBS or diphtheria toxin, respectively. As seen in previous experiments analyzing the potential effect of regulatory T cells on the infiltration of effector T cells into the uveitic iris (Figure 13), and in contrast to the results of Lee et al. (94, 2010), we did not observe a noticeable influence of regulatory T cell presence on the behavior of iris-infiltrating CD11c-YFP$^+$ dendritic cells (Figure 24). This result is open to multiple interpretations: in view of the experimentally-supported view that dendritic cells do not migrate from the eye to the draining lymph node, unlike what is observed in the pancreas (and elsewhere; 231, Melli 2010), it is conceivable that regulatory T cells do not exert the same control over iris-infiltrating CD11c-YFP$^+$ dendritic cells as Lee et al. (94, 2010) report in their diabetes model, owing to the lack of an interaction between these cell types in the lymph node draining the eye. It remains to be seen if this phenomenon will be reproduced by other groups, examining other models of T cell-mediated autoimmune disease. As we observed a significant influx of CD11c-YFP$^+$ dendritic cells into the inflamed iris resulting solely from intravitreal challenge with protein (Figure 21), in the absence of antigen-specific T cells, it is also possible that an antigen-independent aspect of CD11c-YFP$^+$ dendritic cell migration exists that precludes our detecting an effect due to regulatory T cell influence.

In summary, we report on the trafficking of CD11c-YFP$^+$ dendritic cells to the inflamed iris, in response to non-specific stimulation and T cell-mediated inflammation, as reported in other models (EAE: 243, Jain 2010). Using CD11c-YFP$^+$ and Lys-GFP$^+$ reporter animals, we show that an iris-resident macrophage population takes up antigen in the naïve and inflamed iris, not a CD11c$^+$ dendritic cell population as reported elsewhere. We demonstrate, for the first time,

a “sympathizing” effect on CD11c-YFP^{+} iris-resident dendritic cells in the unmanipulated contralateral eye to an antigen-challenged eye. Lastly, we do not observe an influence of regulatory T cells on the invasion of the antigen-challenged iris in a DO11.10 T cell-mediated uveitis model, in contrast to a single previous report using a diabetes model.

Conclusions

The investigation of the role of regulatory T cells in our particular uveitis model that we describe here has produced numerous unexpected findings. Our first step, to determine the baseline presence of regulatory T cells in the naïve murine eye, was undertaken using non-invasive, intravital microscopy to analyze the anterior segment of the eyes of healthy, adult Foxp3-GFP reporter animals. These animals have been used extensively by many laboratories, and have been shown to have regulatory T cells faithfully expressing GFP. Our findings, that Foxp3-GFP$^+$ cells were visible only in the limbus of the eye and nowhere in the intraocular compartment nor in the cornea, contrasts with our findings in the skin and other reports showing regulatory T cells to be present throughout peripheral tissues. This finding is not entirely surprising, as the eye is an immune-privileged organ, with no prior description of lymphocytes within its confines in homeostatic conditions. The only entirely unexpected aspect of these results is the observation of Foxp3-GFP$^+$ cells locating specifically in the limbus, in an age-dependent manner. While the cornea is well known to exhibit immunoprivileged properties, such as its high rate of successful engraftment, we have little reason to believe that these cells contribute substantively to corneal healing. This is due to our observation that tremendous numbers of circulating Foxp3-GFP$^+$ cells were seen to infiltrate the cornea from the circumferential limbal vessels in our uveitis model, perhaps ten-fold greater than the number of limbal-resident Foxp3-GFP$^+$ cells in the naïve eye (unpublished observation). Instead, a more interesting and experimentally-supported hypothesis is that the regulatory T cells present in the corneoscleral limbus localize to that particular site in order to protect the limbal stem cells, which are known to reside there; this relationship has been recently demonstrated for regulatory T cells and hematopoietic stem cells in the bone marrow. In order to investigate this possibility,

the examination of the eyes of scurfy animals (lacking regulatory T cells) may prove useful, as the absence of enhanced protection from inflammatory insult that these limbus-resident regulatory T cells would presumably confer could manifest as a defect in normal corneal homeostasis. However, it is not certain that a defect in limbal stem cell function, of which the readout would be corneal integrity, would be observed in the absence of a direct inflammatory insult to the cornea and/or limbal stem cells. In such a scenario, the scurfy cornea may in fact appear normal or nearly normal. The well-known blepharitis that is present in scurfy animals may be sufficient to provide such an inflammatory insult, and merits further investigation. Other technical approaches to ascertaining the potential role of the limbus-resident regulatory T cells in protecting the limbal stem cells would include local administration of diphtheria toxin in Foxp3-GFP-DTR animals, perhaps crossed with Foxp3-GFP animals, via intravitreal or topical routes, although separating their function from that of circulating regulatory T cells would prove challenging. Along these lines, confirmation of the presence of regulatory T cells in human limbal tissue is of paramount interest, as well as a comparison of the distribution of regulatory T cells in human conjunctivae (bulbar and palpebral) with what we have reported in the murine tissue. Our findings also suggest that the presence of regulatory T cells in other tissues throughout the body, be they immune-privileged such as the CNS and gonads, or sites of presumed significant immunologic activity like other mucosal sites, should be directly determined.

With regards to our main findings, that regulatory T cells in our system do not appear to influence the behavior of effector T nor dendritic cells insofar as we assessed them via intravital microscopy, multiple reasons could explain this result. A principal explanation is that, by using activated effector T cells, we bypassed the stage of regional control exerted over effector T cells

by dendritic cells and regulatory T cells in the lymph node. Thus, our data suggest that the principal function of regulatory T cells in contributing to the resolution of uveitis, as seen in other models, occurs in the lymph node and not in the target tissue. This is somewhat surprising, given the significant numbers of regulatory T cells observed in the iris in our uveitis model, and suggests that either their role *in situ* is negligible, at least in our model of short-lived ocular inflammation, or that other means of control of effector T cells are still functional in the inflamed eye. A well-known intraocular mechanism of minimizing the activity of eye-invasive effector T cells is AICD (activation-induced cell death), wherein the high levels of FasL found in the eye bind to Fas on the surface of infiltrating effector T cells, inducing their apoptosis. Investigating this phenomenon would be straightforward through the use of adoptive transfer with Fas-deficient T cells or using FasL-deficient mice as recipients, although it is uncertain if these animals are available on the BALB/c background (Thomas Ferguson, personal communication). Other efforts to investigate our model using sites besides the eye as the target organ, such as the joint or skin, could shed light on whether AICD contributes significantly to the elimination of the effector T cells. The limited persistence of ovalbumin in our model, which we have observed to remain visible in labeled phagocytic cells for at least one week after intravitreal injection, suggests that loss of antigen may be a factor due to the physiologic turnover of intraocular fluids; other approaches to ensure greater antigen persistence, such as through virus-mediated transduction, may result in prolonged retention of effector T cells and greater inflammation wherin the role of regulatory T cells would be more prominent. A separate approach to identifying a potential role of regulatory T cells in our uveitis model would be the transfer of antigen-specific regulatory T cells concomitantly with the effector T cells, which has proven immunosuppressive in other models, or even to inject them locally. Lastly, examination of the

molecules contributing to the trafficking of regulatory T cells to the eye soon after the induction of uveitis, such as would be possible through cell-sorting and microarray analysis of the isolated cells, would provide useful information regarding the possible existence of an "eye-specific" homing signature for T cells. We have made initial attempts at this investigation, but the yield of isolated cells has been low owing to the laborious task of separating by dissection the intraocular tissues from the conjunctival tissues, where large numbers of effector and regulatory T cells accumulate owing to the uptake of antigen that backflows following intravitreal injection. Alternative approaches using either genetic expression of the target antigen, or viral transduction, may allow for this technical hurdle to be overcome by obviating the need for intravitreal injection of antigen. Once achieved, microarray analysis of the relative expression levels of adhesion molecules and chemokine receptors by iris-invasive regulatory and effector T cells may explain the fairly consistent ratio of these cells that we observed in the uveitic eye and prove informative in directing future investigations into eye-specific biological therapy. Examination of the role of regulatory T cells in other models of T cell-mediated inflammation, particularly in the immune-privileged CNS, may also contribute to our understanding of the function of regulatory T cell in ocular inflammation; in the multiple sclerosis model EAE, regulatory T cells have been shown not to be effective in controlling acute inflammation despite accumulating at the tissue-site in high numbers, in parallel with our findings (244, Korn 2007).

Our principal finding on dendritic cells in our uveitis model is the apparent lack of an influence from regulatory T cells on the dendritic cell invasion into the inflamed iris. This contrasts with a single report of this phenomenon in a diabetes model of T cell-mediated autoimmune pancreatitis. To date, this phenomenon has been neither confirmed nor refuted in the literature, and it will be interesting to see if our results reflect one more particularity of

dendritic cells with respect to the immune-privileged eye, or if this observation is not reproduced in other models of T cell-mediate autoimmune disease. Nonetheless, we describe multiple other novel aspects of dendritic cell behavior in the inflamed iris, namely the lack of significant uptake of labeled antigen and a "sympathizing" response in one eye following inflammatory insult in the contralateral eye. Our observation that dendritic cells rarely appear to take up labeled antigen, whereas an iris-resident macrophage cell population does, stands in contrast to reports of such behavior in the pancreas and iris. Whereas the report on the iris used a different and unconventional technique to identify the dendritic cells, namely *in vivo* immunohistology, the report on the antigen uptake by pancreas-resident dendritic cells used the exact same CD11c-YFP reporter animals as we did, and the same technical assessment via intravital microscopy. Given this commonality, our different results are striking and suggest a tissue-specific effect on dendritic cell function. However, the phenomenon described for pancreas-resident dendritic cells, which we were not able to reproduce in our uveitis system, has been described only once without confirmation nor refutation in the literature, and therefore must be considered with due caution. Nonetheless, it would be informative to investigate the potential effect of the ocular environment on dendritic cell behavior by studying the antigen uptake of iris explants *ex vivo*, to see if they exhibit similar behavior as described in the pancreas. The skin or conjunctiva may also prove to be useful sites for corroborating this phenomenon in non-immunoprivileged tissues, although an initial attempt on our part to look at the uptake of antigen by skin-resident cells via intravital microscopy was not successful owing to the persistence of the injected antigen in the extracellular environment. Examining dendritic cell behavior for this phenomenon in other immune-privileged sites, such as the central nervous system, may prove informative in determining the effect of the local tissue environment, but should probably be reserved for

pursuit after it has been established in other, immune-competent sites. As for the unique "sympathizing" effect that we observe wherein inflammatory insult in one eye leads to increased day-to-day change in the distribution of dendritic cells in the unmanipulated, contralateral eye, the likeliest mechanism that can be invoked to explain this unique phenomenon is one of neuronally-mediated crosstalk between the two eyes, as has been demonstrated previously in a model of retinal injury. It is well known that neurons can detect and influence inflammatory insults by secreting immunologically active peptides. Testing this phenomenon's dependence on neuronal activity would conceivably be possible via pharmacologic, surgical, or genetic manipulation of the potential pathways involved. Furthermore, this phenomenon may provide a useful model for studying neuronal impact on immunologic events, which has been invoked to explain the typical unilateral, alternating, self-limiting presentation of the most common form of uveitis in humans, HLA-B27-associated anterior uveitis. Identifying the exact neuronal mediators involved may shed light on innate immunosuppressive mechanisms that contribute to the resolution of intraocular inflammation.

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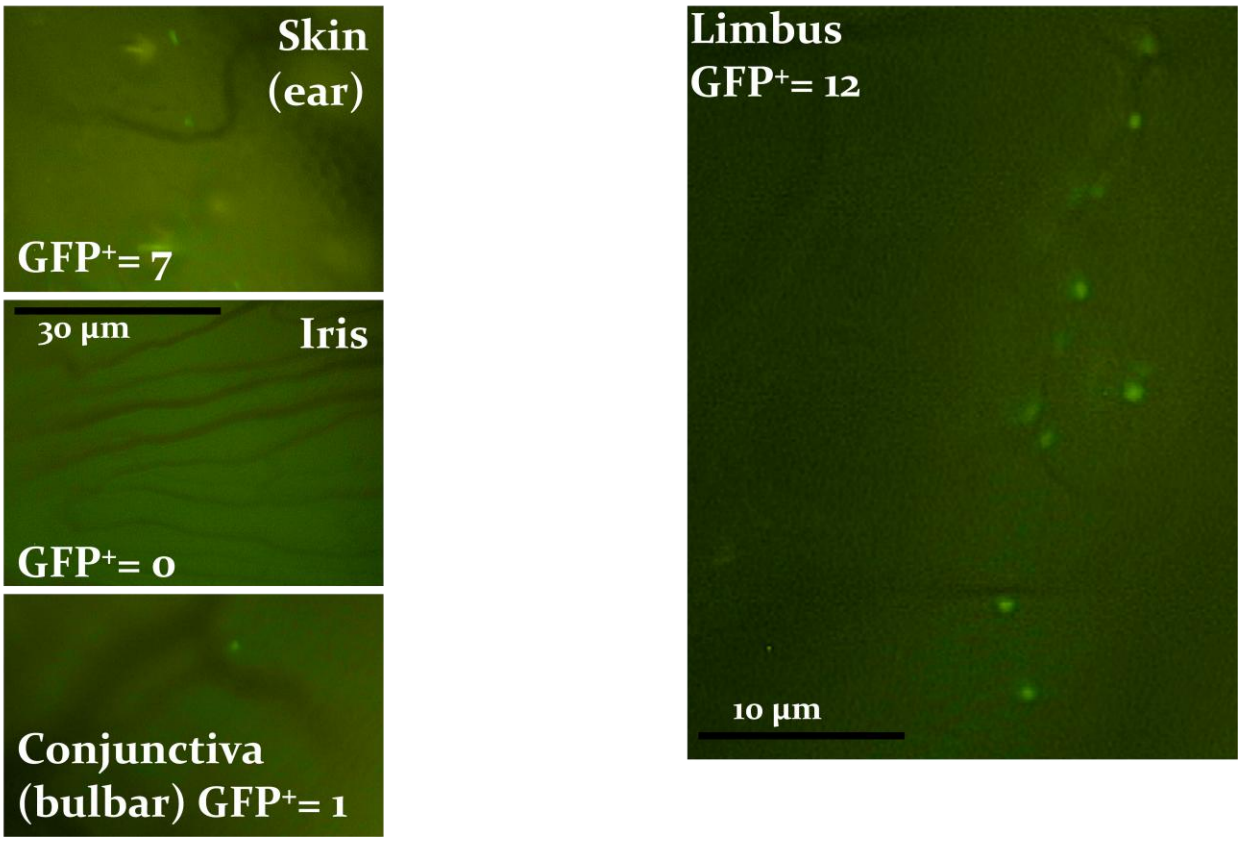

Figure 1. Distribution of Foxp3-GFP$^+$ cells in the skin and anterior segment of the quiescent murine eye of naïve anesthetized animals. Foxp3-GFP$^+$ cells were detected via non-invasive intravital microscopy (200x). In the eye proper, Foxp3-GFP$^+$ cells were present only in the limbal region in naïve murine eyes (right panel). Foxp3-GFP$^+$ cells were readily visible in the ear skin (upper left) and rarely in the bulbar conjunctiva (lower left), but were never seen in the iris (middle left) nor cornea (not shown). Scale bars, 30 µm (left) or 10 µm (right). Representative images are shown of previously unexamined adult (8-12 week old) animals.

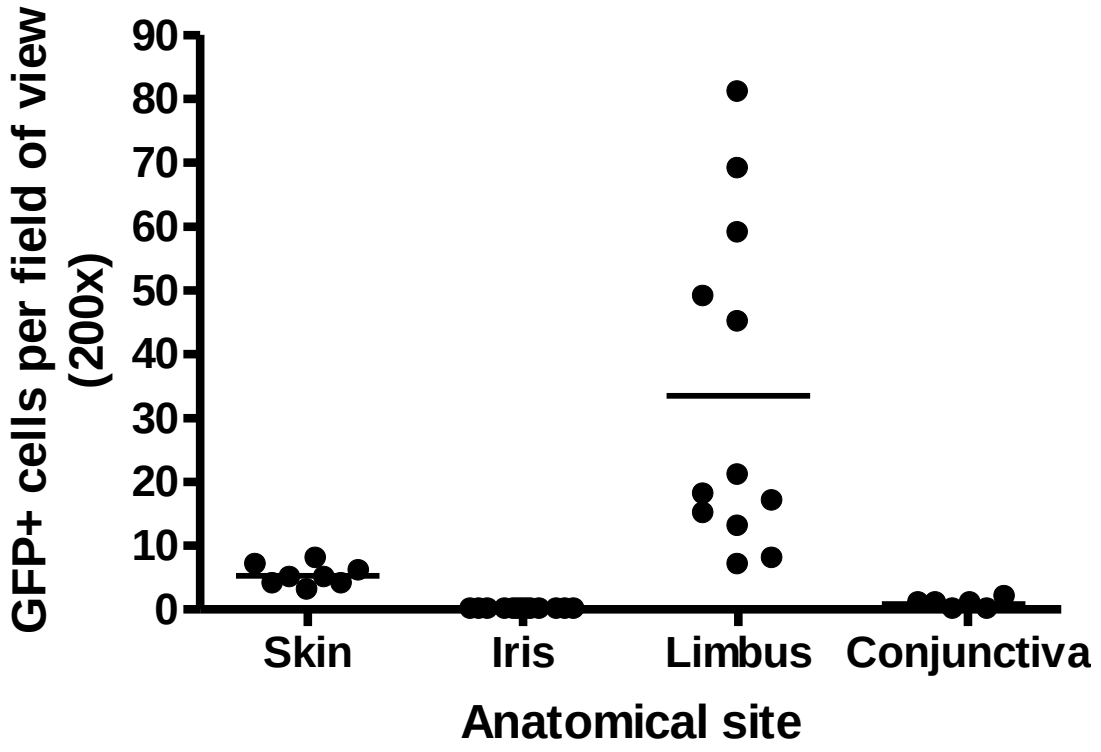

Figure 2. Distribution of Foxp3-GFP$^+$ cells in the skin, bulbar conjunctiva and anterior segment of the quiescent murine eye of anesthetized adult (8-12 week old) animals. Foxp3-GFP$^+$ cells were detected via non-invasive intravital microscopy. In the eye proper, Foxp3-GFP$^+$ cells were present only in the limbal region in naïve murine eyes. Foxp3-GFP$^+$ cells were readily visible in the ear skin and rarely in the bulbar conjunctiva, but were never seen in the iris. Each data point represents an average of at least three fields of view per anatomical site per previously unexamined animal. The data points of the limbus shown here are also depicted in Figure 4.

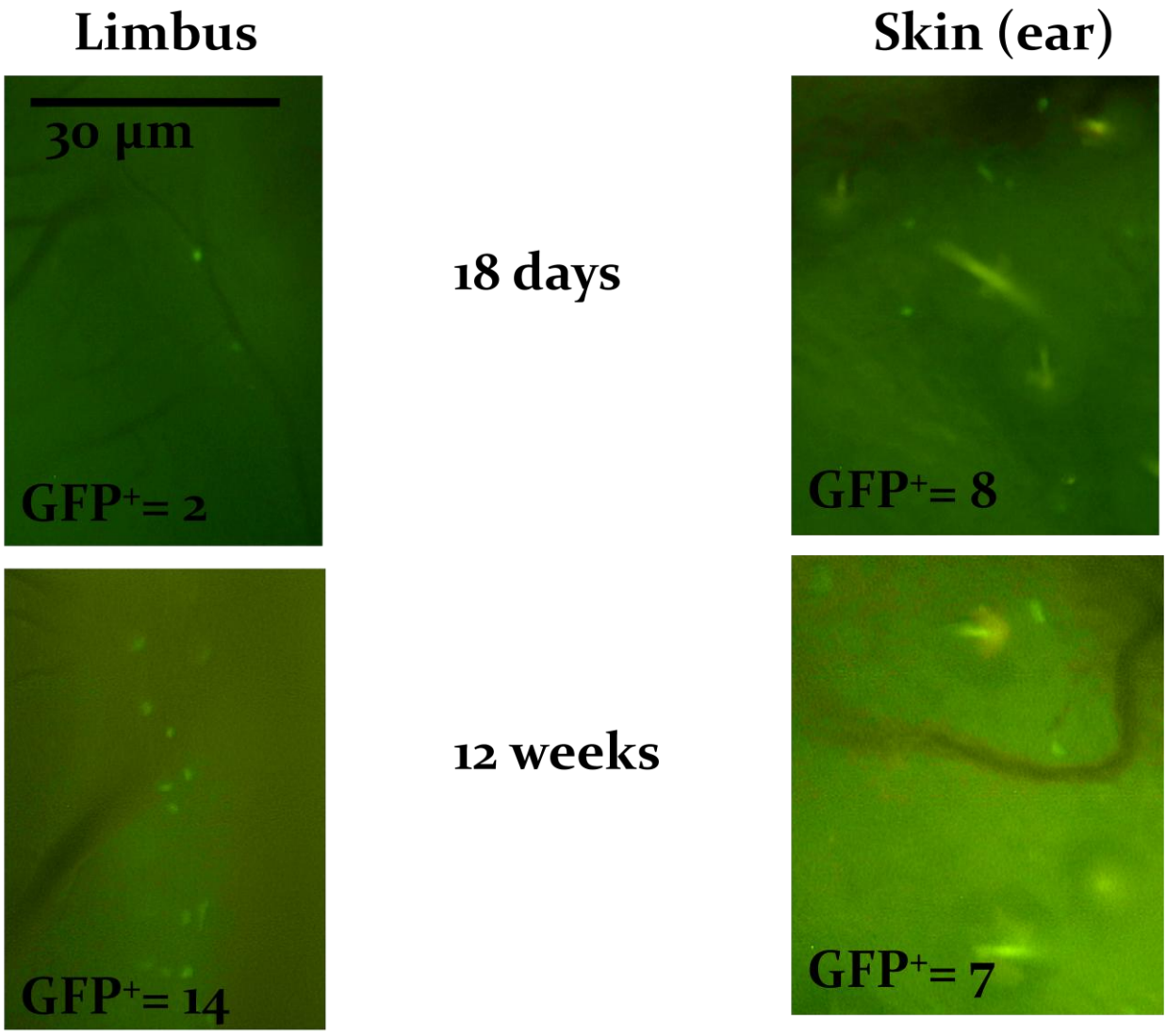

Figure 3. Distribution of Foxp3-GFP$^+$ cells in the quiescent limbus and ear skin of weanling and adult animals. Naïve Foxp3-GFP$^+$ anesthetized animals were examined via non-invasive intravital microscopy (200x) at the indicated ages. Representative images are shown of previously unexamined animals. Foxp3-GFP$^+$ cells were observed to increase in numbers with age in the limbus, but not in the skin.

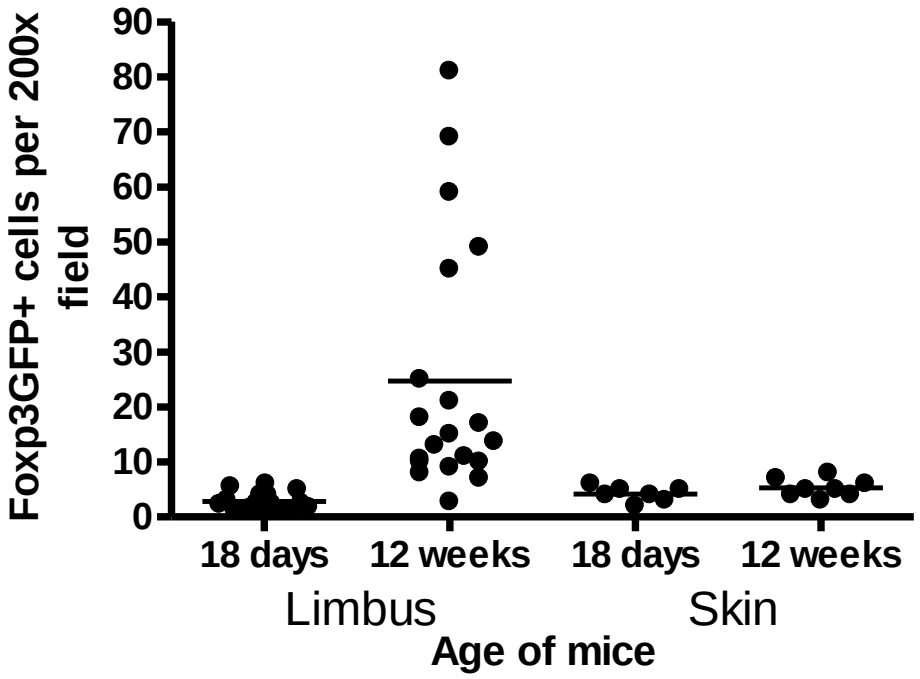

Figure 4. Distribution of Foxp3-GFP^{+} cells in the limbus and ear skin of weanling and adult animals. Naïve Foxp3-GFP^{+} anesthetized animals were examined via non-invasive intravital microscopy at the indicated ages. Each animal was being examined for the first time. Each data point is an average of cell counts of at least three fields of view per tissue examined per individual mouse. Foxp3-GFP^{+} cells were observed to increase in numbers with age in the limbus, but not in the skin. The adult data points depicted here are the same as those depicted in Figure 2.

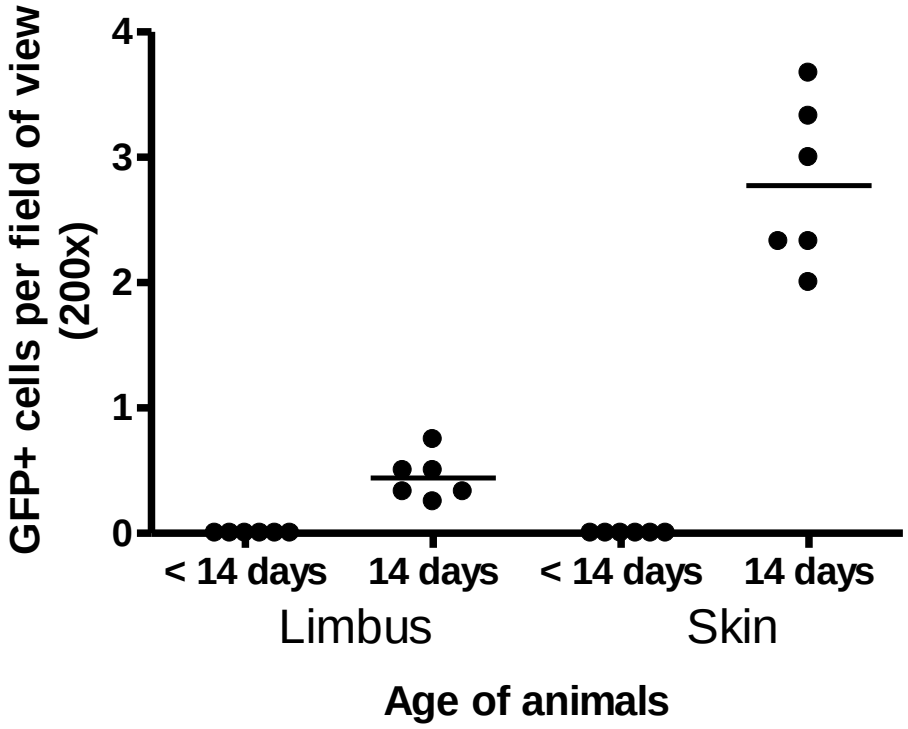

Figure 5. Initial observation of Foxp3-GFP$^+$ cells in the peripheral tissues of young Foxp3-GFP reporter animals. The skin and limbus of naïve, anesthetized Foxp3-GFP$^+$ reporter animals were examined via non-invasive intravital microscopy prior to (8 days of age), concomitant with (12-13 days of age), and following (14 days of age) eye opening. Each animal was being examined for the first time. At 8 days of age, the skin of the tip of the tail and heel of the footpad were examined, whereas the footpad was examined at 12 days of age. The ear skin and footpad were examined at 14 days of age. Each data point is an average of cell counts of at least three fields of view per tissue examined per individual mouse. Foxp3-GFP$^+$ cells were observed to appear at the same time point, 14 days of age, in the limbus and in the skin.

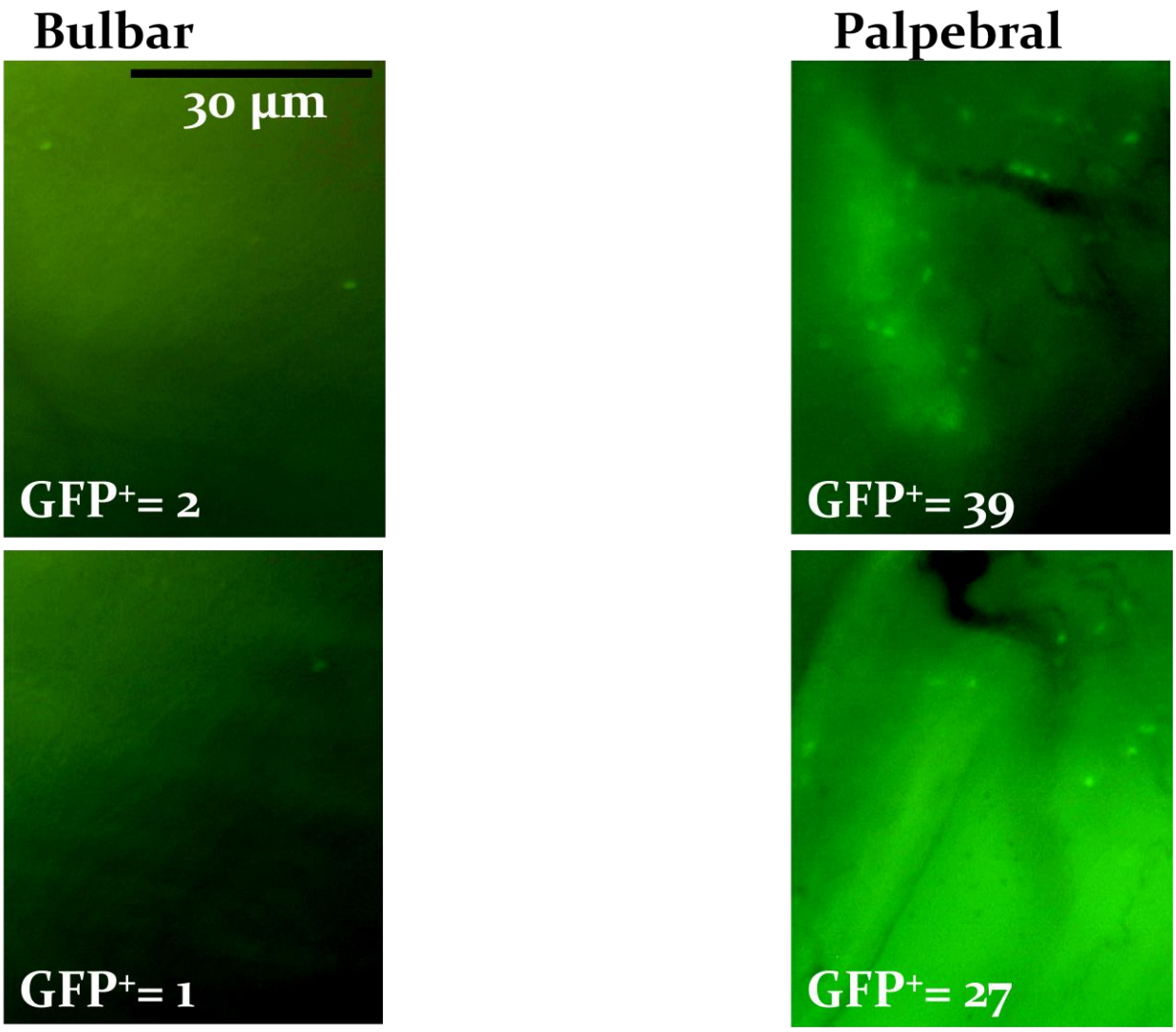

Figure 6. Distribution of Foxp3-GFP$^+$ cells in the conjunctivae of naïve adult animals. The conjunctivae of naïve, anesthetized Foxp3-GFP reporter adult (8-12 weeks) animals were examined initially via non-invasive intravital microscopy and, subsequently, *ex vivo* using the same microscope (200x). Representative images are shown of previously unexamined animals. Foxp3-GFP$^+$ cells were observed in high numbers in the palpebral, but not in the bulbar, conjunctivae.

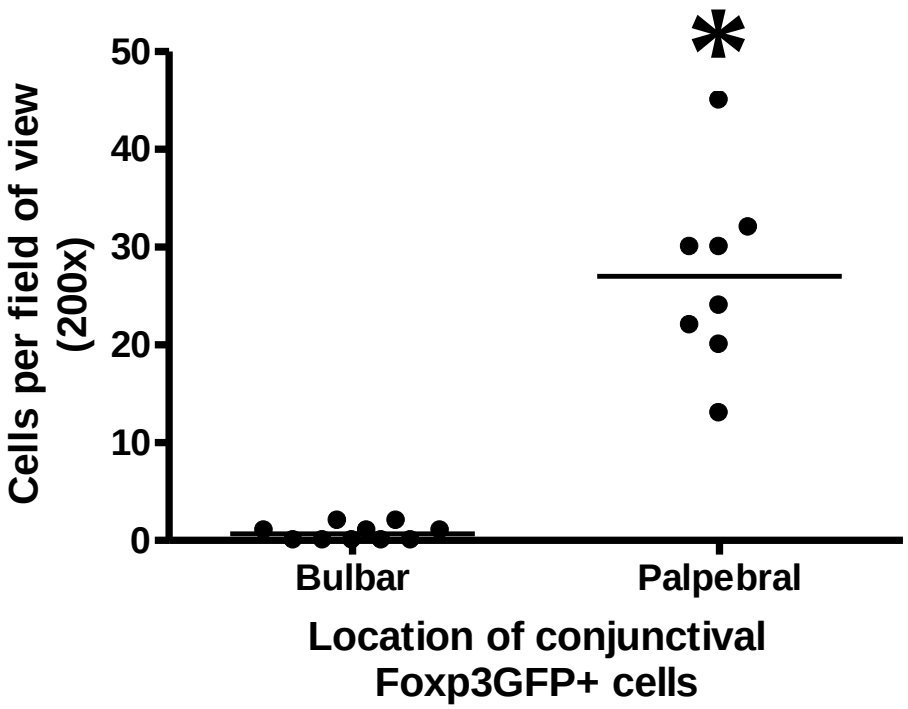

Figure 7. Distribution of Foxp3-GFP^{+} cells in the conjunctivae of naïve adult animals. The conjunctivae of naïve, anesthetized Foxp3-GFP reporter adult (8-12 weeks) animals were examined initially via non-invasive intravital microscopy and, subsequently, *ex vivo* using the same microscope. Each animal was being examined for the first time. Each data point is an average of cell counts of at least three fields of view per tissue examined *in vivo* and *ex vivo* per individual mouse. *$p<0.05$. Foxp3-GFP^{+} cells were observed in high numbers in the palpebral, but not in the bulbar, conjunctivae.

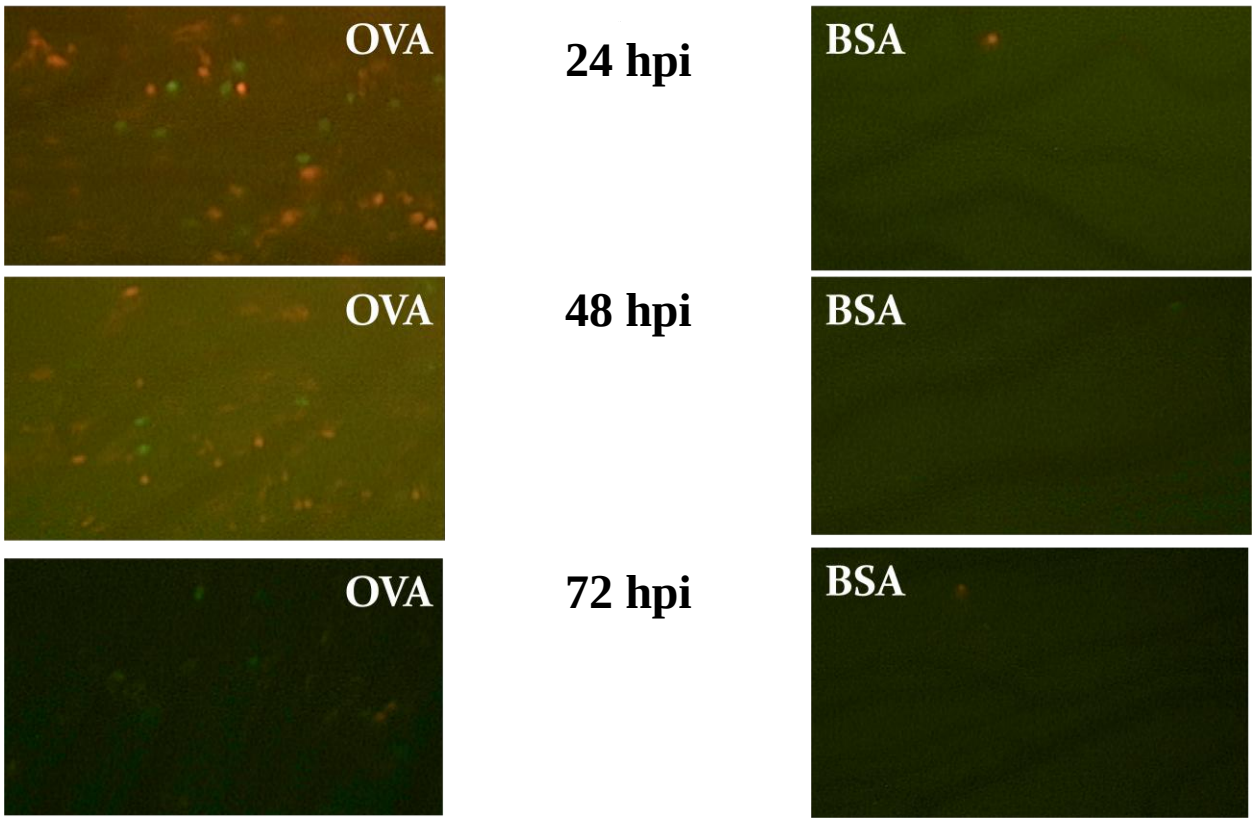

Figure 8. Presence of red fluorescent DO11.10 effector T cells and Foxp3-GFP^{+} cells in the irides of uveitic eyes. The irides of Foxp3-GFP reporter animals were imaged via non-invasive, intravital microscopy (200x) at the stated time points after transfer of fluorescently red-labeled DO11.10 cells and intravitreal challenge with ovalbumin (OVA) or a control protein (BSA). High numbers of red and green cells were visible in the specific antigen-challenged, but not control, eyes at 24 and 48 hours before decreasing significantly by 72 hours.

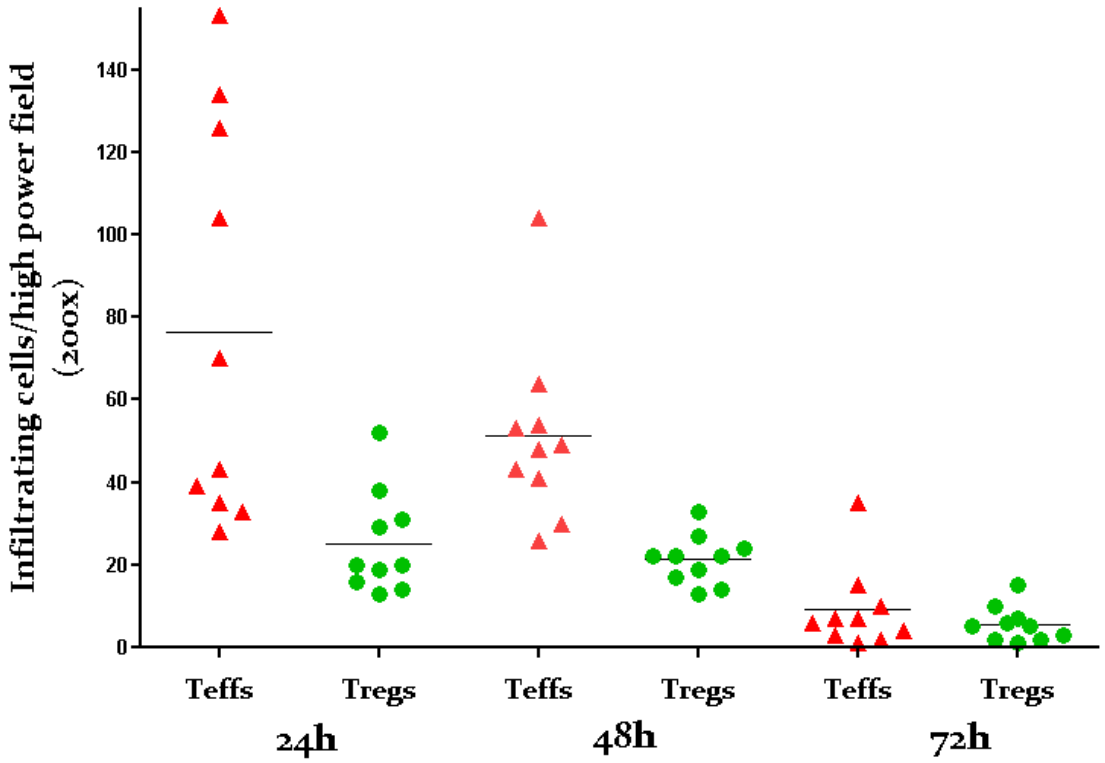

Figure 9. Presence of red fluorescent DO11.10 effector T cells and Foxp3-GFP^{+} cells in the irides of specific antigen-challenged (uveitic) eyes. The irides of Foxp3-GFP reporter animals were imaged via non-invasive, intravital microscopy at the stated time points after transfer of fluorescently red-labeled DO11.10 cells and intravitreal challenge with ovalbumin (OVA). Each data point represents an average of the numbers of cells of at least three fields of view in a given eye from three separate experiments. High numbers of red and green cells were visible in the specific antigen-challenged, but not control, eyes at 24 and 48 hours before decreasing significantly by 72 hours.

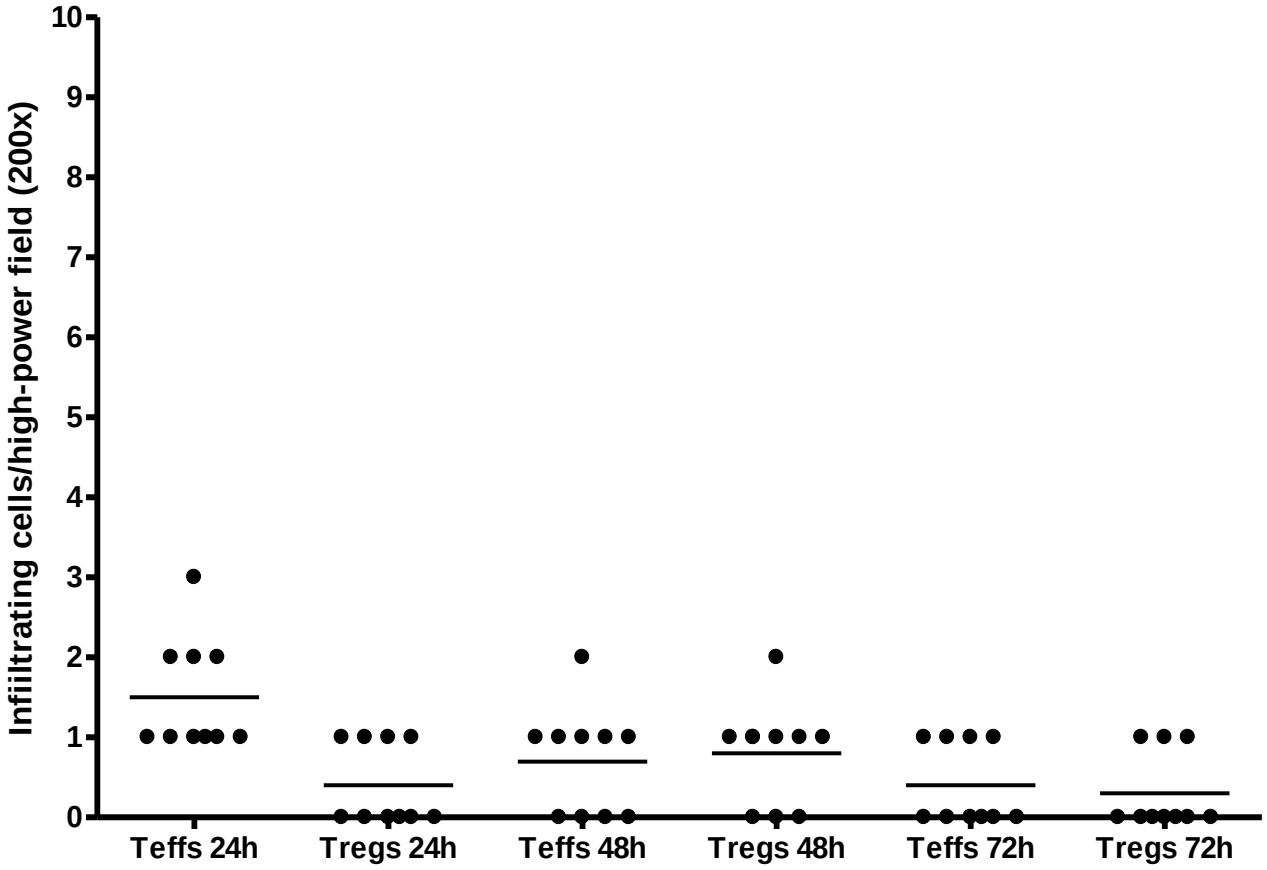

Figure 10. Presence of DO11.10 effector T cells and Foxp3-GFP^{+} cells in the irides of control eyes. The irides of Foxp3-GFP reporter animals were imaged via non-invasive, intravital microscopy at the stated time points after transfer of fluorescently red-labeled DO11.10 cells and intravitreal challenge with control protein (BSA). Each data point represents an average of the numbers of cells of at least three fields of view in a given eye from three separate experiments. High numbers of red and green cells were visible in the specific antigen-challenged, but not control, eyes at 24 and 48 hours before decreasing significantly by 72 hours.

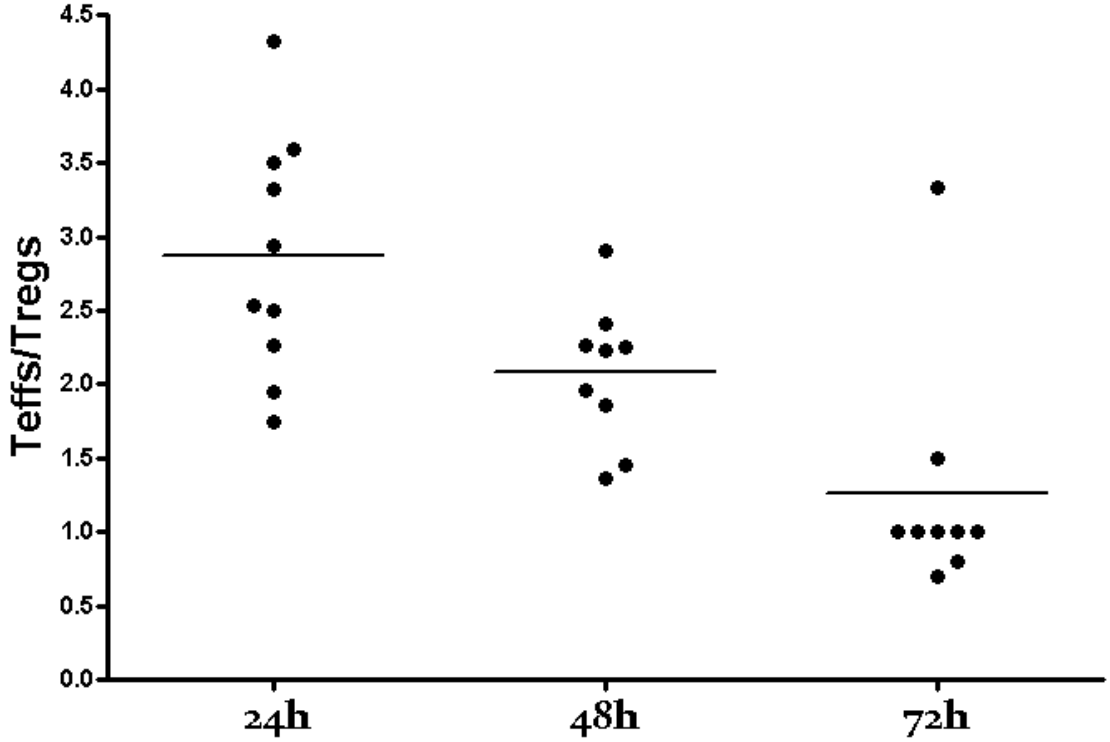

Figure 11. Ratio of adoptively transferred DO11.10 effector T cells to host regulatory T cells visible in the irides of specific antigen-challenged eyes. The irides of Foxp3-GFP^{+} reporter animals were imaged via non-invasive, intravital microscopy at the stated time points after transfer of fluorescently red-labeled DO11.10 cells and intravitreal challenge with ovalbumin. Each data point represents an average of the ratio of cells of at least three fields of view in a given eye from three separate experiments. DO11.10 effector T cells exhibited a ratio of approximately 2-3:1 to host regulatory T cells in the irides of specific antigen-challenged eyes at 24 to 48 hours after injection, before cell populations decreased substantially.

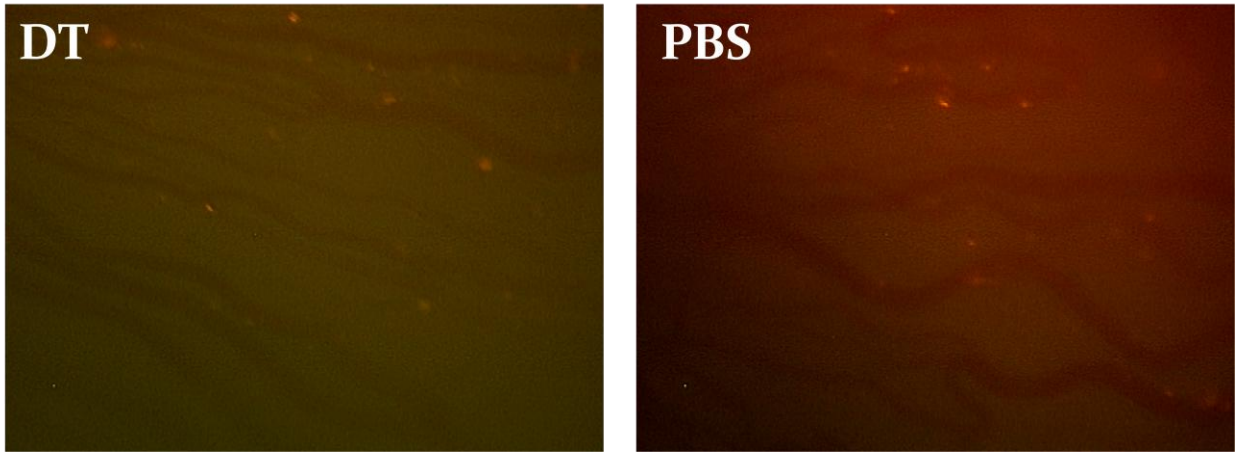

Figure 12. Presence of red fluorescent DO11.10 effector T cells in the irides of specific antigen-challenged (uveitic) eyes of Treg-depleted (or control) Foxp3-GFP-DTR animals. The irides of Foxp3-GFP-DTR animals were imaged via non-invasive, intravital microscopy (200x) at 24 hours after transfer of fluorescently red-labeled DO11.10 cells and intravitreal challenge with ovalbumin. Recipient animals had been pretreated with diphtheria toxin (DT) to deplete Foxp3-GFP-DTR cells, or with control (PBS), at 48 and 24 hours prior to adoptive transfer and intravitreal challenge. DO11.10 red effector T cells were visible in the irides of specific antigen-challenged eyes of both DT-treated and control animals in high numbers at 24 hours after injection.

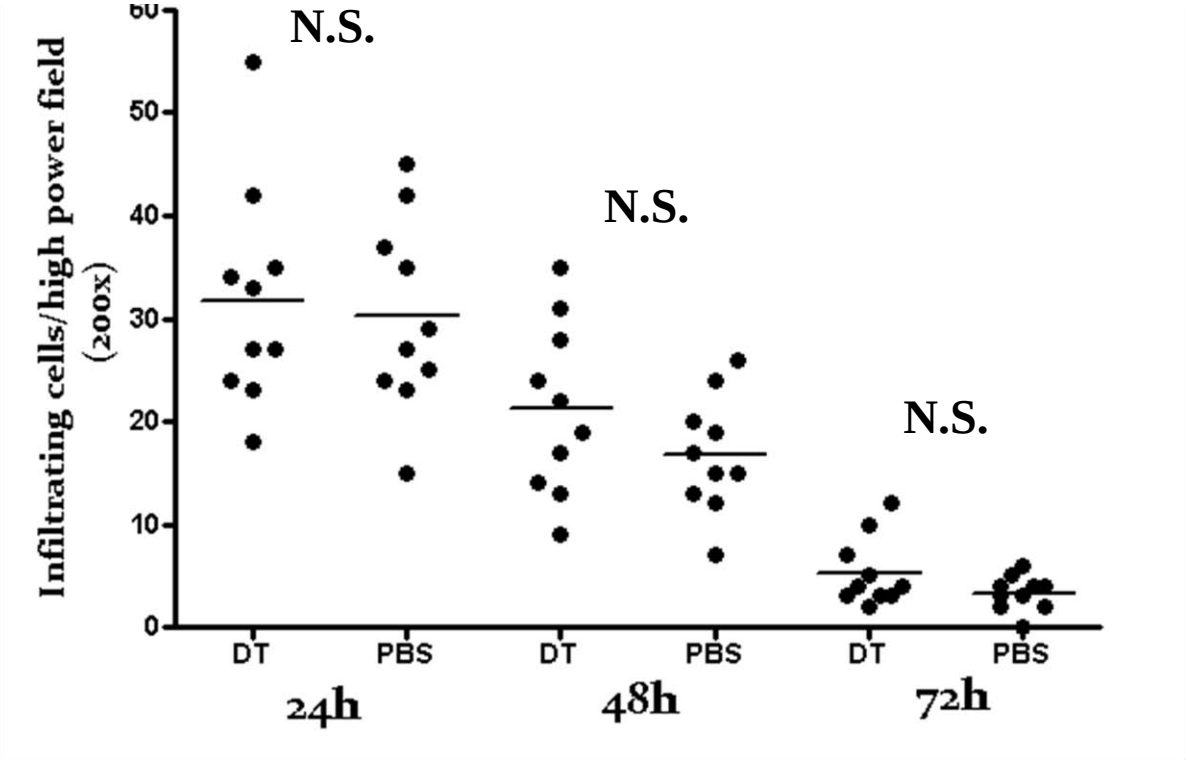

Figure 13. Presence of red fluorescent DO11.10 effector T cells in the irides of specific antigen-challenged (uveitic) eyes of Treg-depleted (or control) Foxp3-GFP-DTR animals. The irides of Foxp3-GFP-DTR animals were imaged via non-invasive, intravital microscopy at the stated time points after transfer of fluorescently red-labeled DO11.10 cells and intravitreal challenge with ovalbumin or BSA (control eye). Recipient animals had been pretreated with diphtheria toxin (DT) to deplete Foxp3-GFP-DTR cells, or with control (PBS), at 48 and 24 hours prior to adoptive transfer and intravitreal challenge. Ablation of Foxp3-GFP-DTR cells was confirmed by flow cytometry of peripheral blood and/or intravital assessment of the presence of GFP^+ cells. Each data point represents an average of the numbers of cells of at least three fields of view in a given eye from three separate experiments. N.S.= $p>0.05$. DO11.10 red effector T cells were visible in the irides of specific antigen-challenged eyes of both DT-treated and control animals in high numbers at 24 hours after injection, with no statistically significant difference.

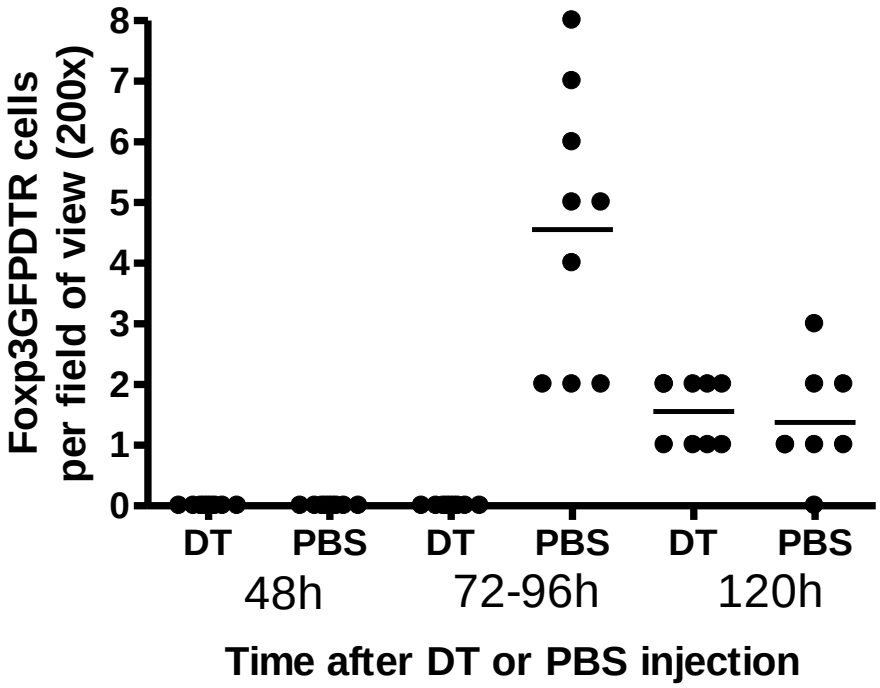

Figure 14. Presence of visible Foxp3-GFP^{+} cells in the limbus of specific antigen-challenged (uveitic) eyes of Treg-depleted (or control) Foxp3-GFP-DTR animals. The limbi of Foxp3-GFP-DTR animals were imaged via non-invasive, intravital microscopy after transfer of fluorescently red-labeled DO11.10 cells and intravitreal challenge with ovalbumin. Recipient animals had been pretreated with diphtheria toxin (DT) to deplete Foxp3-GFP-DTR cells, or with control (PBS), at 48 and 24 hours prior to adoptive transfer and intravitreal challenge. Each data point represents the numbers of cells of at least three fields of view in a given eye of three separate experiments. The presence of Foxp3-GFP^{+} cells in the limbi of specific antigen-challenged control, but not Treg-depleted, eyes for the first several days after uveitis induction demonstrates the depletion efficiency of this methodology.

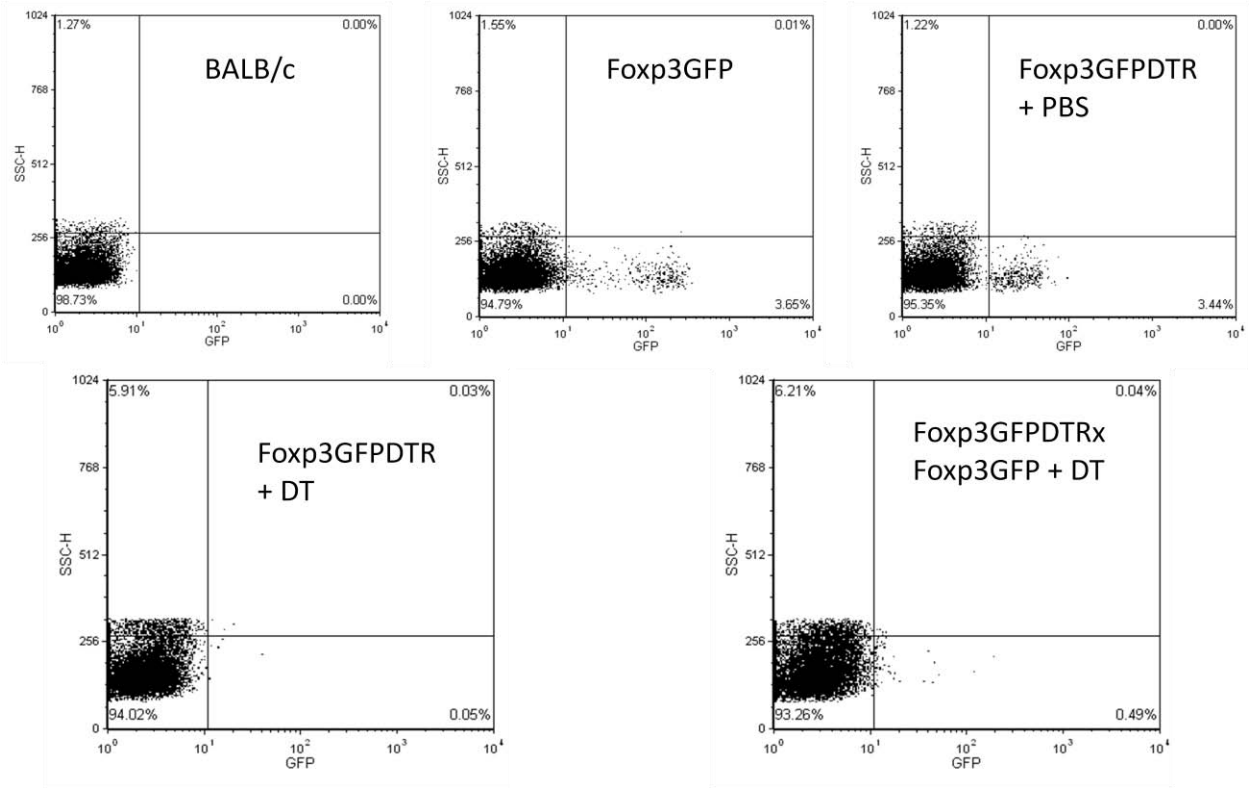

Figure 15. Assessment of the depletion of Tregs in Foxp3-GFP-DTR animals. Representative flow cytometry analysis of peripheral blood lymphocytes from the indicated adult mice following two days of intraperitoneal treatment with diphtheria toxin (DT) or control (PBS): 1) naïve BALB/c 2) naïve Foxp3GFP reporter animals 3) Foxp3-GFP-DTR animals treated with PBS 4) Foxp3-GFP-DTR animals treated with DT 5) Foxp3-GFPDTRxFoxp3GFP animals treated with DT. Foxp3-GFP^{+} cells are much brighter than Foxp3-GFP-DTR^{+} cells per flow cytometry, corroborating the *in vivo* findings, and the latter are efficiently depleted by DT.

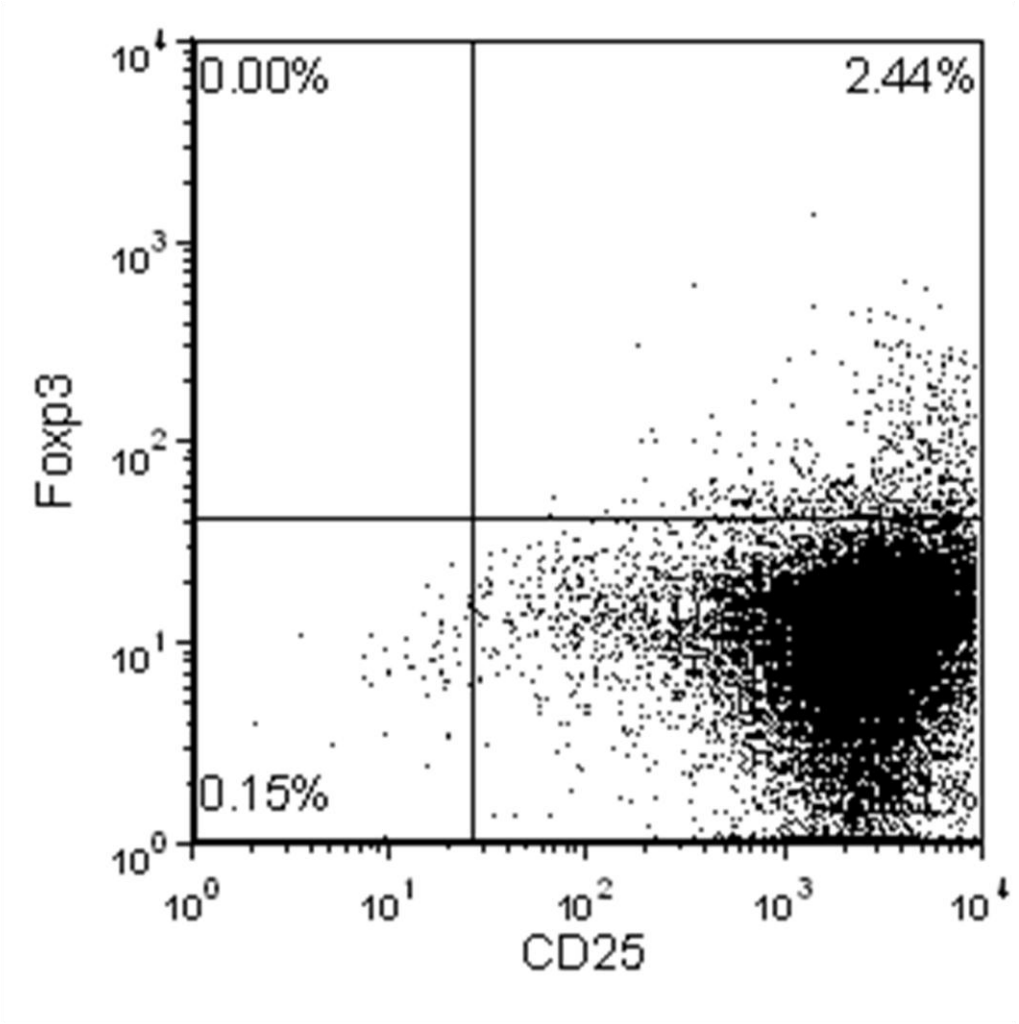

Figure 16. Assessment of the presence of $CD4^+CD25^+Foxp3^+$ cells in the inoculum of *in vitro* cultured DO11.10 cells prior to adoptive transfer. Representative flow cytometric analysis of a four day-old splenic culture of DO11.10 cells. A small percentage of T cells in the inoculum express regulatory T cell markers, suggesting an unlikely role for these cells in suppressing the inflammatory event.

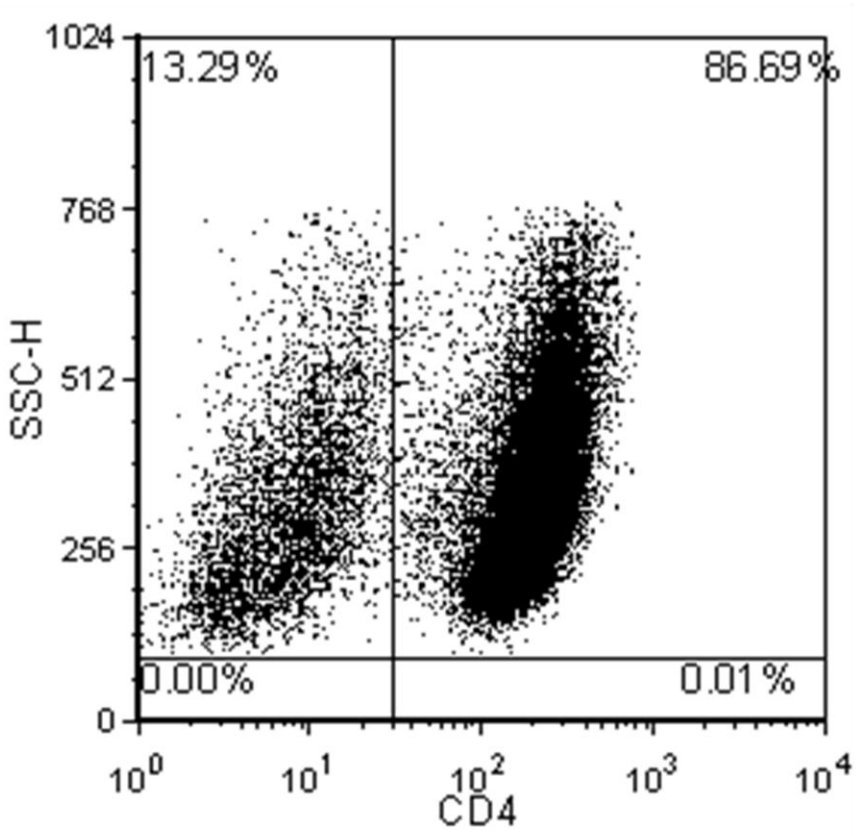

Figure 17. Assessment of the presence of $CD4^+$ cells in the inoculum of *in vitro* cultured DO11.10 cells prior to adoptive transfer. Representative flow cytometric analysis of a four day-old splenic culture of DO11.10 cells. The vast majority, nearly 90%, of the transferred cells were $CD4^+$ T cells.

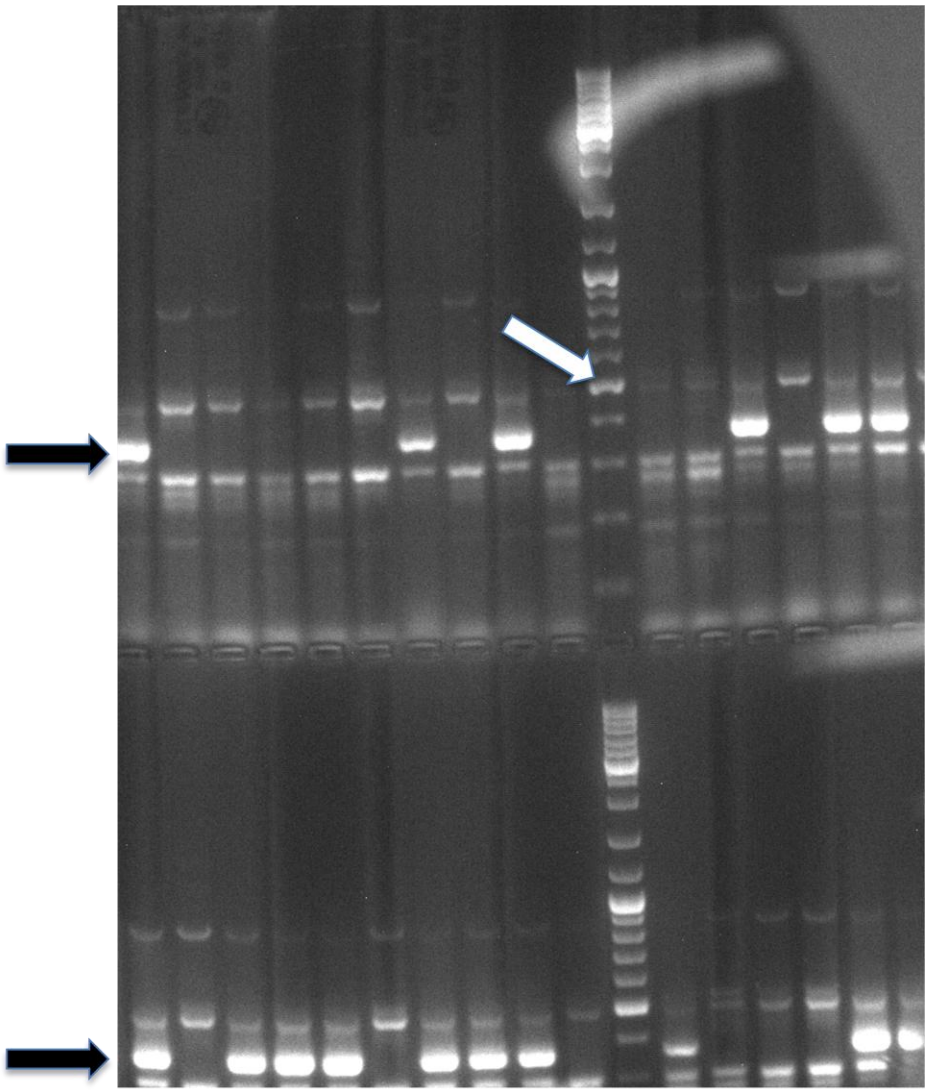

Figure 18. Genotyping of Foxp3-GFP-DTR animals. Representative gel showing the PCR genotyping results of Foxp-GFP-DTR progeny. The DTR specific band, migrating at around 350bp, is indicated by the black arrows. As a reference, the 500bp marker is indicated by the white arrow. The first two lanes on the left on the top and bottom are positive and negative controls, respectively.

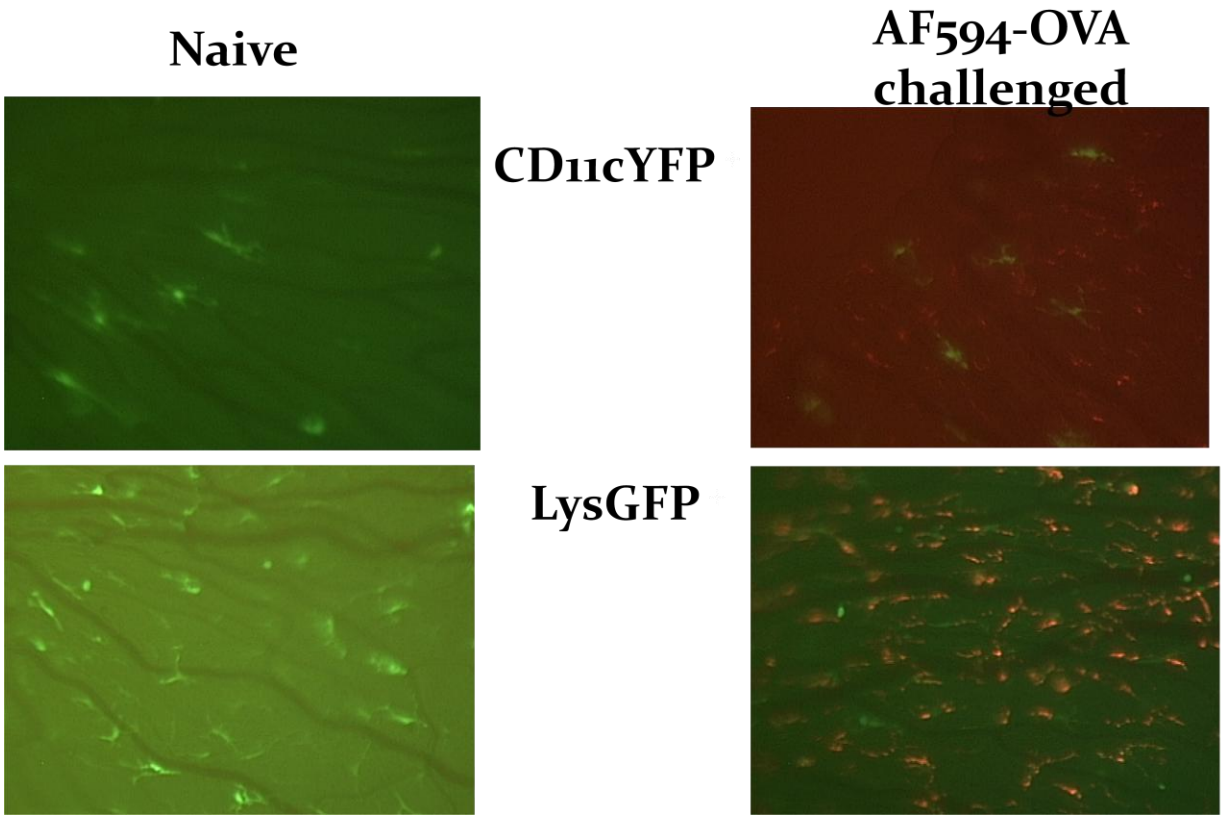

Figure 19. Uptake of fluorescently labeled protein by iris-resident CD11c-YFP^{+} (dendritic cells) and LysGFP^{+} (macrophages) cells. Irides of naïve, adult (8-12 week old) CD11c-YFP^{+} and LysGFP^{+} transgenic animals were imaged via non-invasive, intravital microscopy (left, 200x) prior to being challenged via intravitreal injection with 2 μl of 10 μg/μL of Alexa Fluor 594 (red)-conjugated chicken ovalbumin. 24 hours later, irides of anesthetized, antigen-challenged animals were re-imaged via intravital microscopy (right). CD11c-YFP^{+} dendritic cells rarely, unlike LysGFP^{+} cells, appear to take up fluorescently labeled antigen in the murine iris.

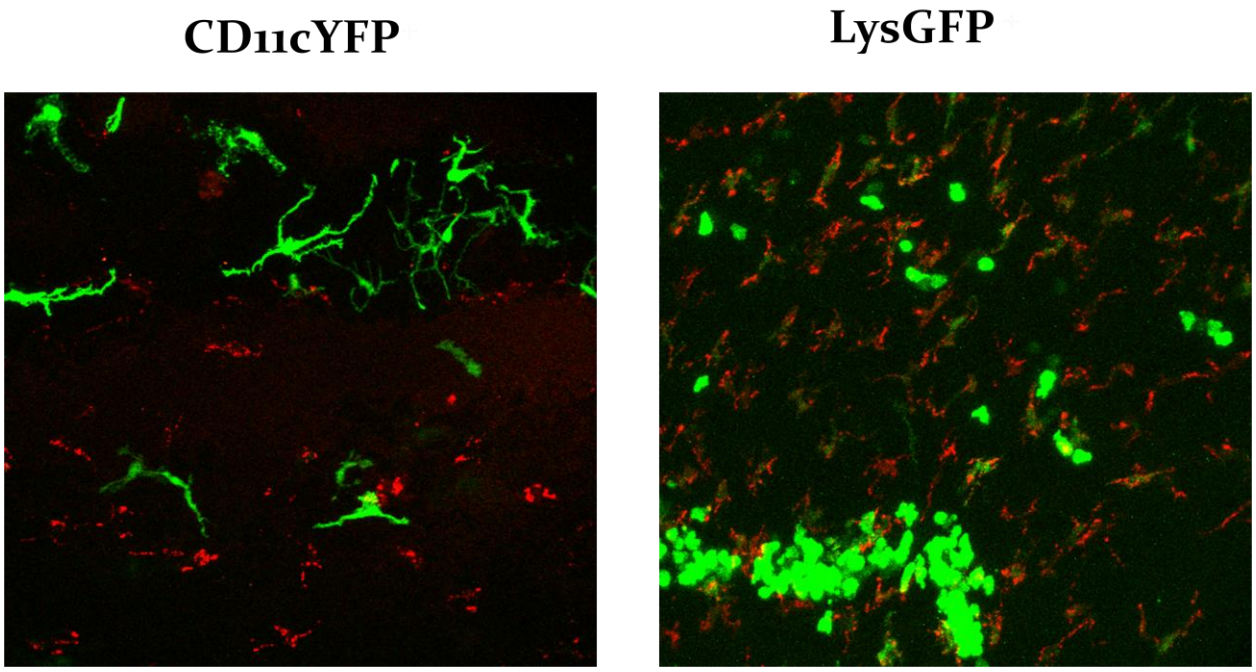

Figure 20. Uptake of fluorescently labeled protein by iris-resident CD11c-YFP^{+} and LysGFP^{+} phagocytic cells. Naïve, adult (8-12 week old) CD11c-YFP^{+} and LysGFP^{+} transgenic animals were challenged via intravitreal injection with 2 μl of 10 μg/μL of Alexa Fluor 594 (red)-conjugated chicken ovalbumin. 24 hours later, irides from enuclated, fixed eyes were imaged via confocal microscopy (200-400x). The bright, round cells in the LysGFP^{+} image that do not take up antigen are neutrophils. CD11c-YFP^{+} dendritic cells rarely, unlike LysGFP^{+} cells, appear to take up fluorescently labeled antigen in the murine iris.

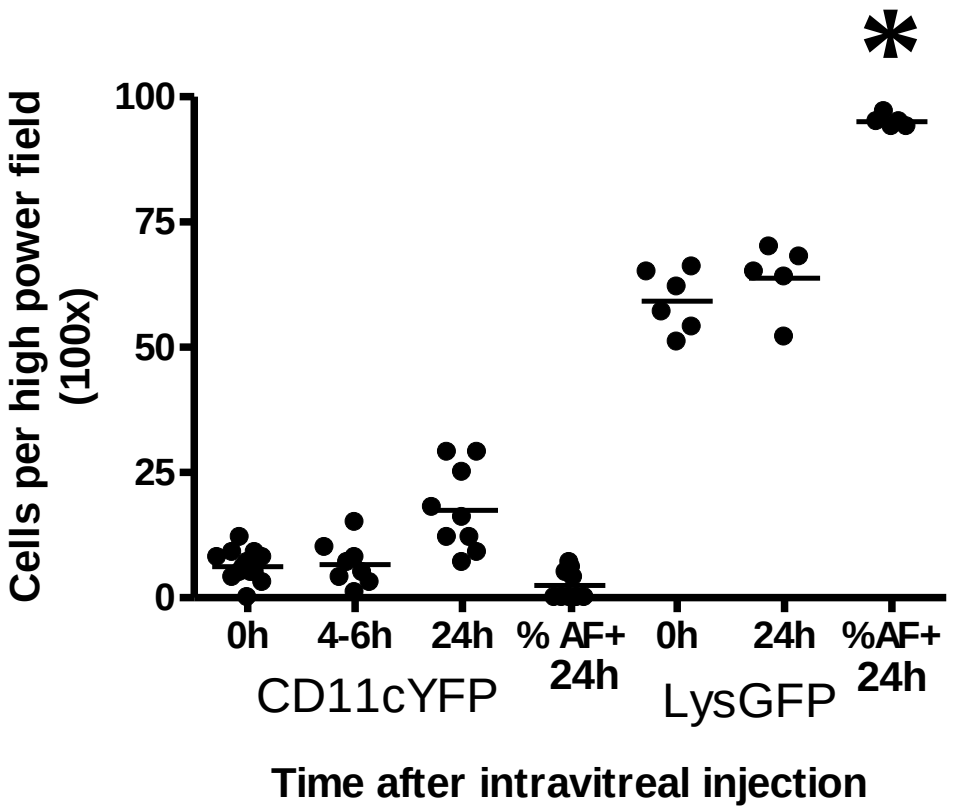

Figure 21. Uptake of fluorescently labeled protein by iris-resident CD11c-YFP$^+$ (dendritic cells) and LysGFP$^+$ (macrophages) cells. Irides of naïve, adult (8-12 week old) CD11c-YFP$^+$ and LysGFP$^+$ transgenic animals were imaged via non-invasive, intravital microscopy (200x) prior to being challenged via intravitreal injection with 2 μl of 10 μg/μL of Alexa Fluor (AF) 594 (red)-conjugated chicken ovalbumin. 24 hours later, irides of anesthetized, antigen-challenged animals were re-imaged via intravital microscopy. CD11c-YFP$^+$ or LysGFP$^+$ cells were considered AF positive based on overlap of fluorescence in intravital and confocal images. The difference between the percentages of AF$^+$-CD11c-YFP$^+$ and LysGFP$^+$ cells was statistically significant (*$p<0.05$). CD11c-YFP$^+$ dendritic cells rarely, unlike LysGFP$^+$ cells, appear to take up fluorescently labeled antigen in the murine iris. Additionally, CD11c-YFP$^+$ dendritic cells, unlike LysGFP$^+$ cells, increase in number after injection of fluorescently labeled antigen in the murine iris.

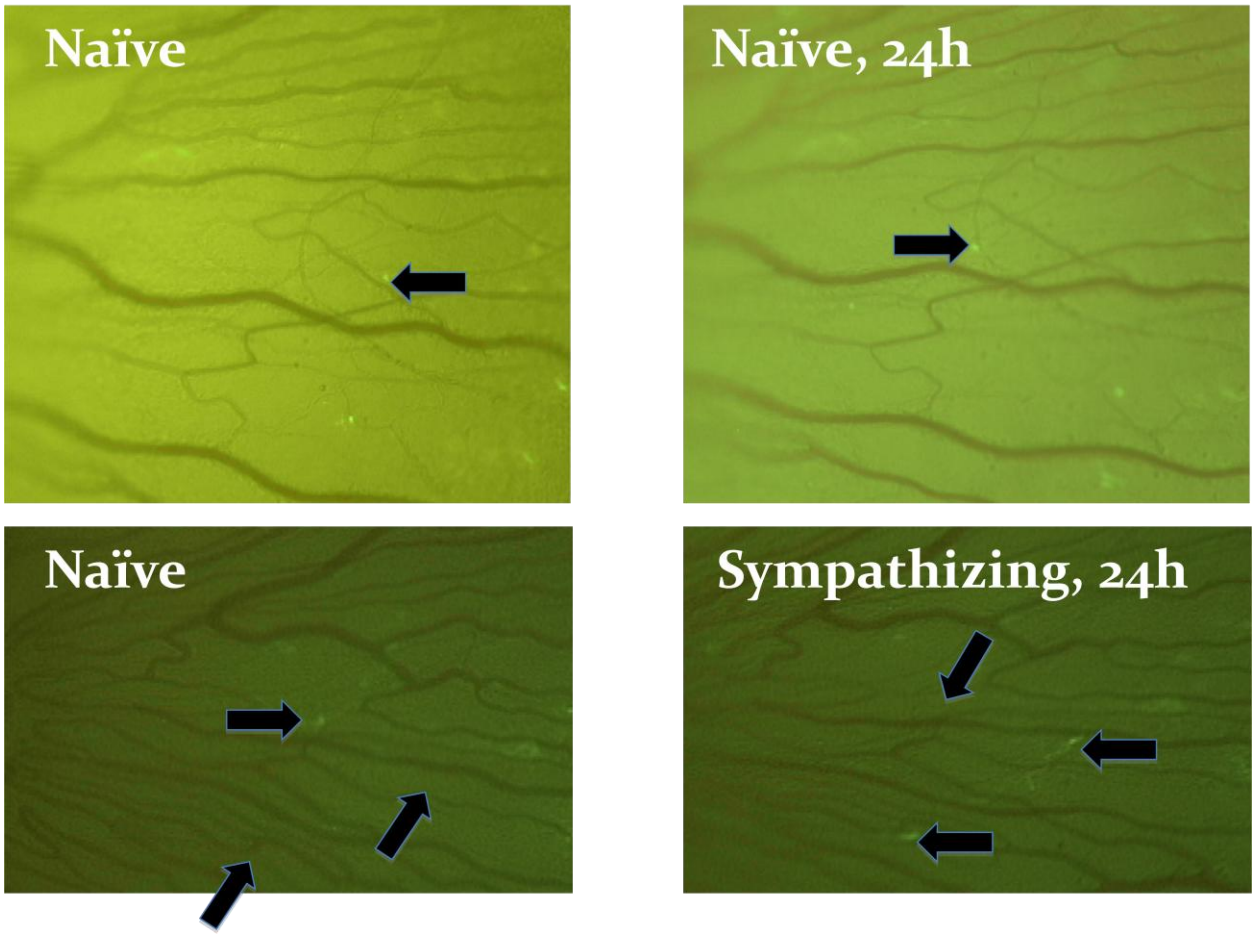

Figure 22. Examination of motility of iris-resident CD11c-YFP^{+} dendritic cells in naïve and sympathizing eyes. The irides of naïve, adult (8-12 week old) previously unexamined CD11c-YFP^{+} transgenic animals were imaged via non-invasive, intravital microscopy (100x). The experimental animal was subsequently challenged via intravitreal injection with 2 μl of 10 μg/μL of Alexa Fluor 594 (red)-conjugated chicken ovalbumin in one eye, whereas the control animal was untouched. 24 hours later, the iris from the contralateral eye was re-imaged via intravital microscopy. The presence of new CD11c-YFP^{+} cells or absence of previously viewed cells was noted, as well as total cell numbers. Arrows indicate cells that were not present at the same location at the other time point and their corresponding location in the other image. CD11c-YFP^{+} dendritic cells rarely exhibited day-to-day changes in their distribution in the quiescent iris, but were exhibited significant changes in a sympathizing response.

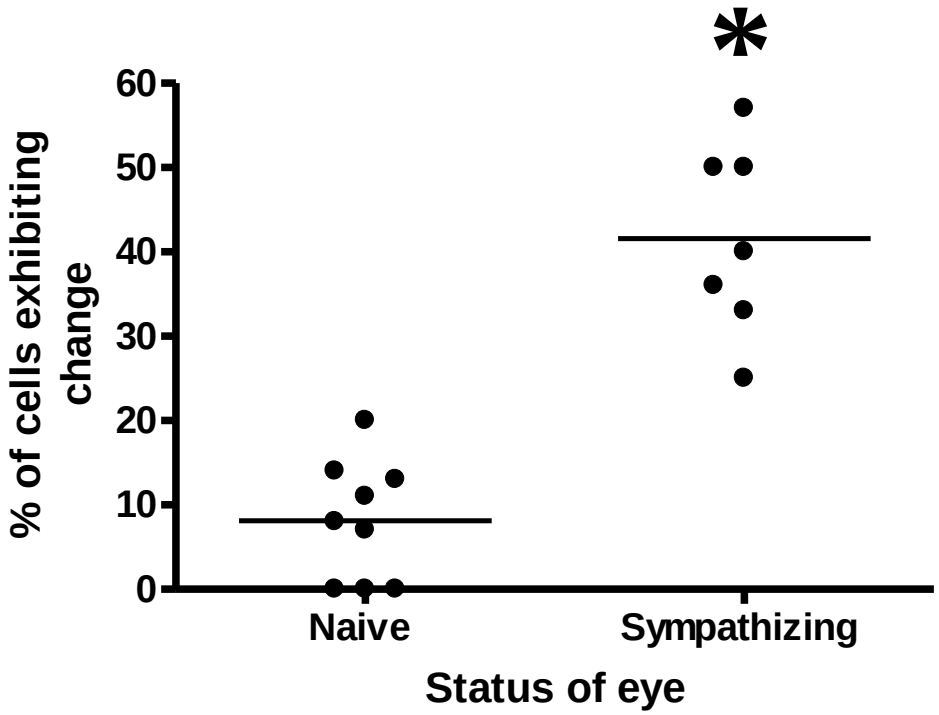

Figure 23. Examination of kinetic activity of iris-resident CD11c-YFP$^+$ dendritic cells in naïve and sympathizing eyes. The irides of naïve, adult (8-12 week old) previously unexamined CD11c-YFP$^+$ transgenic animals were imaged via non-invasive, intravital microscopy (100x). The experimental animal was subsequently challenged via intravitreal injection with 2 μl of 10 μg/μL of Alexa Fluor 594 (red)-conjugated chicken ovalbumin in one eye, whereas the control animal was untouched. 24 hours later, the iris from the contralateral (sympathizing) eye was re-imaged via intravital microscopy. The presence of new CD11c-YFP$^+$ cells or absence of previously viewed cells was noted, as well as total cell numbers. Percentage was determined as the number of cells differing between the two images per total of visible cells. *$p<0.05$. CD11c-YFP$^+$ dendritic cells rarely exhibited day-to-day changes in their distribution in the quiescent iris, but were exhibited significant changes in a sympathizing response.

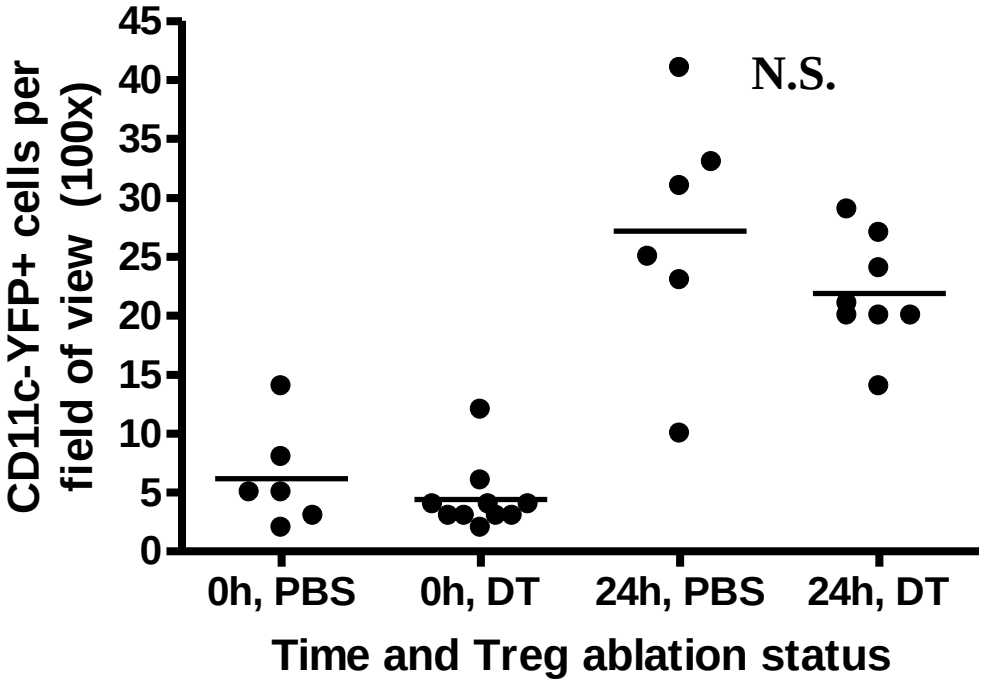

Figure 24. Presence of CD11c-YFP$^+$ dendritic cells in the irides of specific antigen-challenged (uveitic) eyes of Treg-depleted (or control) Foxp3-GFP-DTRxCD11cYFP$^+$ animals following adoptive transfer of antigen-specific DO11.10 cells. The irides of Foxp3-GFP-DTRxCD11cYFP$^+$ animals animals were imaged via non-invasive, intravital microscopy (100x) at the stated time points, both prior to and following adoptive transfer of fluorescently red-labeled DO11.10 cells and intravitreal challenge with ovalbumin or BSA (control eye). Recipient animals had been pretreated with diphtheria toxin (DT) to deplete Foxp3-GFP-DTR cells, or with control (PBS), at 48 and 24 hours prior to adoptive transfer and intravitreal challenge. Ablation of Foxp3-GFP-DTR cells was confirmed by intravital assessment of the presence of GFP$^+$ cells. Each data point represents an average of the numbers of cells of at least three fields of view in a given eye from three separate experiments. N.S.= $p>0.05$. CD11c-YFP$^+$ dendritic cells were visible in the irides of specific antigen-challenged eyes of both DT-treated and control animals in high numbers at 24 hours after injection, with no statistically significant difference.