ROLE OF IL-17A AND ACAID IN THE MAINTENANCE OF

CORNEAL IMMUNE PRIVILEGE

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To my parents, Grotati and Antoine, for all of their support and
for instilling in me a passion for science and academics

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by

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The University of Texas Southwestern Medical Center at Dallas, 2011

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The human cornea, specifically the endothelial cell layer, has little to no regenerative potential. Accordingly, the eye has evolved multifactorial mechanisms that limit local immunogenic inflammation and trauma to the corneal endothelium. This local immune deviation is achieved through multiple anatomical, physiological, and immunoregulatory features intrinsic to the ocular environment that allow the cornea to enjoy an immune privileged status. Corneal immune privilege allows first time recipients of corneal allografts to enjoy a 90% success rate in the absence of systemic immunosuppressive drugs and without prior tissue typing. The myriad of

immunoregulatory components that conspire to maintain the immune privilege of the cornea can be assigned to one of two extensively studied subsets. They include: (a) immunosuppressive molecules and membrane molecules present in the aqueous humor and on the surface of corneal cells layers, and (b) cell-mediated regulatory mechanisms such as anterior chamber-associated immune deviation (ACAID) that suppress systemic immune responses. This dissertation proposal investigates the role of a newly identified T cell subset, the Th17 $CD4^+$ T cell lineage and its production of the cytokine, IL-17A in modulating corneal immune privilege and the outcome of keratoplasty. Previously, the Th17 T cell subset and its pro-inflammatory cytokine, IL-17A had been associated with the pathogenesis of several autoimmune diseases and had been implicated in cardiac, lung, and renal allograft rejections. Interestingly, in contrast to the classical paradigms that suggest that $CD4^+$ T cells mediate graft rejection, the IL-17A produced by $CD4^+$ T cells appears to be necessary for corneal allograft survival. My investigations led me to two distinct mechanisms via which IL-17A promotes corneal immune privilege. The results indicate that IL-17A is required for inhibiting the generation of an allospecific Th2 $CD4^+$T cell subset that can independently exacerbate corneal allograft rejection. Simultaneously, IL-17A is also required for the functioning of $CD4^+CD25^+$ T regulatory cells (Tregs) that suppress alloimmune responses directed against the corneal transplant. Finally, by comparing and contrasting the cellular and cytokine requirements of corneal immune privilege and ACAID, these investigations unveil the independent mechanisms operating in the establishment of these respective phenomena. These findings redefine the role of IL-17A as a cytokine essential for the maintenance of corneal immune privilege and establish a new paradigm whereby interplay between IL-17A and $CD4^+CD25^+$ Tregs is necessary for survival of corneal allografts.

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PRIOR PUBLICATIONS

Cunnusamy K., Chen P.W., Niederkorn J.Y. IL-17 Promotes Immune Privilege of Corneal Allografts. Journal of Immunology, 185(8):4651-8, Oct 2010.

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AC	Anterior chamber
AH	Aqueous humor
Abs	Antibodies
ACAID	Anterior chamber-associated immune deviation
AHR	Allergic airway hyperreactivity
APC	Allophycocyanin
APCs	Antigen presenting cells
BAX	Bcl-2-associated X protein
BCL-2	B-cell lymphoma 2
BCL-xL	B-cell lymphoma-extra large
CD	Cluster of differentiation
CGRP	Calcitonin gene-related peptide
CFSE	Carboxyfluorescein succinimidyl ester
CNS	Central nervous system
CRPs	Complement regulatory proteins
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte Antigen-4
DCs	Dendritic cells
DTH	Delayed-type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FasL	Fas Ligand

FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box P3
GITR	Glucocorticoid-induced tumor necrosis factor receptor family-related gene
GITRL	GITR ligand
GVHD	Graft versus host disease
HBSS	Hanks' balanced salt solution
IC	Intracameral
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-gamma
IL	Interleukin
IP	Intraperitoneally
IV	Intraveneously
KO	Knockout
LAT	Local adoptive transfer
LCs	Langerhans cells
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
mIH	Minor histocompatibility complex
MIF	Macrophage migration inhibitory factor
MLR	Mixed lymphocyte response
MOMP	Mitochondrial outer membrane permeabilization
MRT	Mean rejection time
MST	Median survival time

mTGF-β1	Membrane Transforming growth factor-beta-1
PBS	Phosphate buffered saline
PD-L1	Programmed cell death ligand-1
PE	Phycoerythrin
PercP	Peridinin Chlorophyll Protein Complex
NK	Natural killer cells
qPCR	Quantitative real-time PCR
rm	Recombinant mouse
RPMI	Roswell Park Memorial Institute medium
SC	Subcutaneous
sFasL	Soluble Fas Ligand
SOM	Somatostatin
STAT6	Signal Transducer and Activator of Transcription-6
TCRs	T cell receptors
TGF-β	Transforming growth factor-beta-1
Th	T helper
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
Treg	T regulatory cell
VIP	Vasoactive intestinal peptide
VH	Vitreous humor
αMSH	Alpha-melanocyte stimulating hormone
ºC	Degrees Celsius

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

Anatomy of the Eye

The eye is the peripheral organ for vision by which humans obtain more than 80% of the information from the external world (1). The primary function of this organ is to transmit and convert external visual stimuli in the form of photons into chemical and electrical energy that can then be channeled to the brain via the optic nerve for processing by the visual cortex. Photons penetrating the eye first traverse the anterior segment of the eye which consists of the cornea, iris, lens, ciliary body, and the anterior chamber (AC) (**Fig. 1**). The lens further focuses the light rays and projects the image through the semi-viscous vitreous humor of the posterior segment onto the retina. The image formed on the retina is then sent via the optic nerve to the brain where perception of color, contrast, depth, and form occurs.

The Cornea

The cornea is the gateway for external images entering into the eye and provides approximately 70% of the refractive power of the eye (1). Light rays entering the eyes are initially refracted off the corneal surface which consists of three major layers (**Fig. 1**). The anterior surface of the tissue is made up of the corneal epithelium which is 5 to 6 cells deep and makes up 10% of the total thickness of the cornea (2). Three cell types populate the epithelium. Superficial cells make up the initial two to three outermost layers of the cornea. The superficial cells are joined by desmosomes and tight junctional complexes that prevent penetration of substances through the intracellular spaces. Beneath the superficial cells are two to three layers of wing cells followed by a single layer of basal cells which is adherent to the basement membrane adjacent to the Bowman's layer. Only cuboidal, columnar basal cells have

proliferative activity. The daughter cells stemming from the basal cells gradually emerge to the anterior surface of the cornea, first differentiating into wing cells and then subsequently into superficial cells. The process of differentiation takes about 7 to 14 days before the superficial cells are desquamated into the tear film, thereby allowing a constant turnover of the cell layer.

The next layer of the cornea that the photons encounter in their path is the stroma. The corneal stroma is separated from the corneal endothelium by the basement membrane and Bowman's layer, and makes up 90% of the cornea's volume. The characteristic features of the cornea such as its physical strength, constancy of shape, and transparency are principally based on anatomic and biochemical characteristics of the corneal stroma. The corneal stroma primarily consists of extracellular matrices, mainly collagen and glycosaminoglycans, with keratocytes (corneal fibroblasts) and nerve fibers making up only 2% to 3% of the volume of the stroma. Glycosanimoglycans have the capacity to absorb and retain large amounts of water. Thus, critical regulation of corneal stroma hydration needs to be maintained in order to retain transparency. This function is primarily discharged by the Descemet's membrane and the third layer of the cornea, the corneal endothelium.

Descemet's membrane is the basement membrane of the corneal endothelium and is a deposition of collagenous protein. Physical stress can lead to the rupture of the membrane which is followed by aqueous humor penetration into the corneal stroma, leading to stromal edema and corneal opacity. Consisting of a single layer covering the posterior surface of the Descemet's membrane, the polygonal endothelial cells are essential to maintaining normal stromal deturgescence. The endothelial cells are abundant in organelles and are very active in metabolism and secretion. Endothelial cells contain ion transport systems that counteract infiltration of water into the stroma. Na^+/K^+ ATPases, sodium/hydrogen exchangers, and carbon

dioxide to bicarbonate conversion within the endothelial cells maintain constant osmotic gradients that promote outflow of water. Corneal endothelial cells do not proliferate in humans and the only recourse to maintaining deturgescense of the stroma in the event of endothelial cell loss, involves the neighboring cells enlarging and spreading to cover the defective area.

The Aqueous Humor

Light rays initially refracted at the corneal surface are directed into the AC, which is the fluid-filled space between the cornea and the iris. In humans, the AC is filled with 250 µl of aqueous humor (AH) and the turnover rate for the fluid is approximately 1.5 µl/minute (3). AH is primarily produced by the ciliary body and leaves the AC via the trabecular meshwork whose cells form a filter with a decreasing pore size as the canal of Schlemm is approached. Efferent channels from Schlemm's canal conduct aqueous humor into the venous system. The composition of the AH is similar to that of plasma except for the much higher concentrations of ascorbate, pyruvate, and lactate and lower concentrations of protein, urea, and glucose. In addition to providing an avenue for nutrient and metabolic exchange between the avascular tissues of the cornea and lens, the aqueous humor regulates intraocular pressure and also contains several anti-inflammatory and immunosuppressive molecules that can suppress harmful immune responses that can lead to irreparable injury to innocent bystander cells (4).

The Iris and the Lens

The iris is the next AC structure the photons encounter. The iris is an extension of the ciliary body and divides the aqueous compartment into an anterior and a posterior chambers. The iris forms a diaphragm with an adjustable aperture, the pupil, whose size controls the amount of light transmitted through the lens. In contrast to the cornea, the iris is heavily pigmented and vascularized. The next structure the photons encounter in their path is the crystalline lens. The lens is located behind the iris and pupil in the anterior compartment of the eye and consists of an

asymmetric oblate spheroid that is devoid of nerves, blood vessels, or connective tissue. Lens transparency is primarily dependent on the densely packed crystallins which have been shown to be immunogenic (5). The lens is a vital refractive element of the human eye and by the process of accommodation, focuses images onto the retina.

Posterior Segment of the Eye

Once across the lens, the light rays penetrate into the posterior compartment of the eye which consists of the hyaloid membrane (outer surface of the vitreous), vitreous humor, retina, and optic nerve. Photons first travel through the vitreous humor (VH) which consists of a gelatinous body that makes up two-thirds of the volume and weight of the eye. It occupies the space bounded by the lens, retina, and optic disk and confers the spherical shape to the eyeball. 99% of the vitreous consists of water and the remaining 1% is made up of two highly hydrophilic components: collagen and hyaluronic acid. The choroid lies between the retina and the sclera, and is highly vascularized, and provides oxygen and nourishment to the outer layers of the retina. The light rays eventually complete their journey on the retina, which is a light-sensitive tissue lining the inner surface of the eye. The retina is a highly complex tissue consisting of ten distinct layers whose purpose is to process light signals and transmit them to the brain.

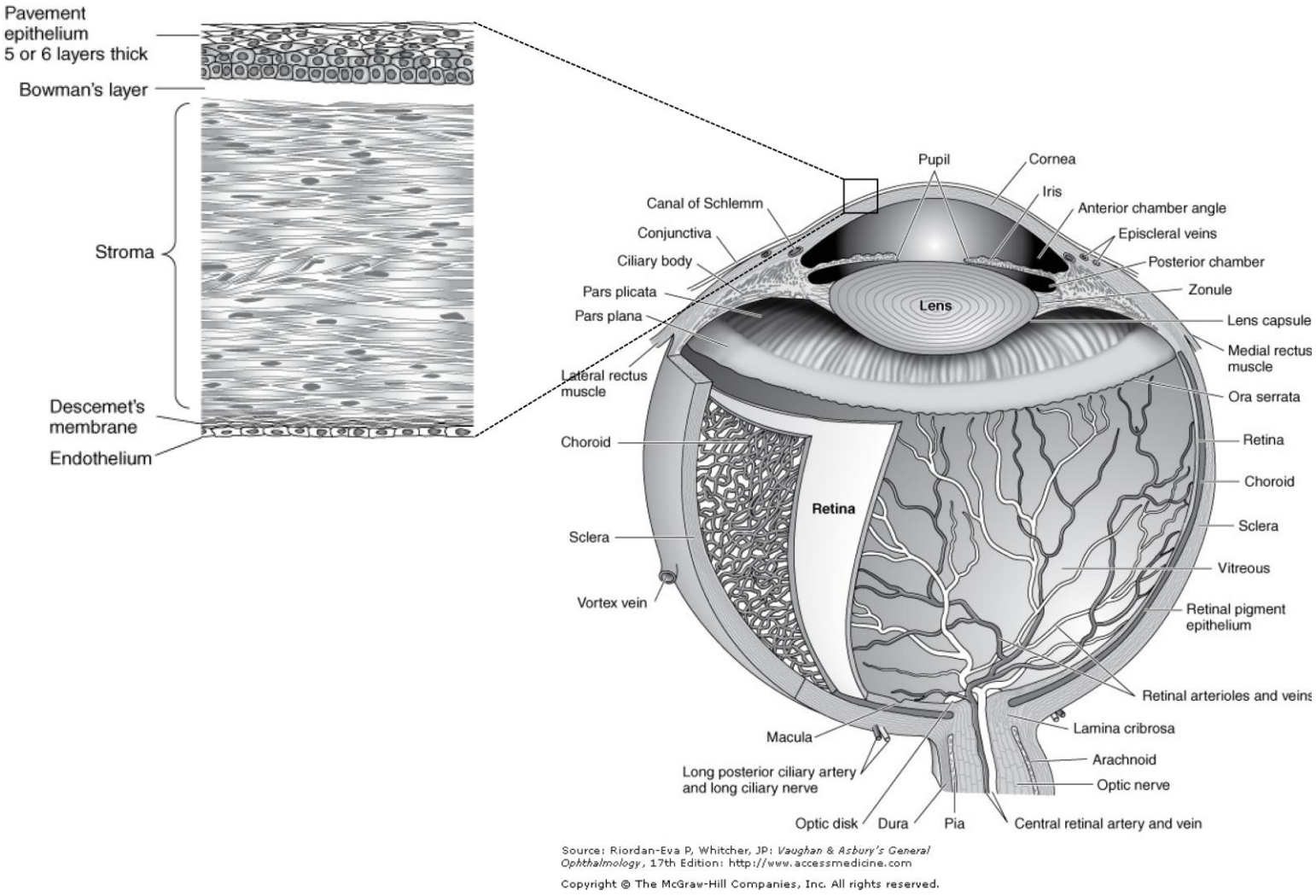

Figure 1. Anatomy of the eye

History of Corneal Transplantation

Three centuries ago, Erasmus Darwin, the grandfather of Charles Darwin was among the few who conceived that successful transplantation of corneal tissues could be a reality. It took an additional century before the idea materialized and the first experimental corneal transplantation was attempted. In 1835, using a pet gazelle as a recipient, Bigger reported the first ever described keratoplasty procedure (6). The next documented attempt was made by Kissam in 1838 who grafted a pig cornea onto a human subject with 4 interrupted sutures, and horrifyingly, without the use of anesthesia (7)**.** As any surgical procedure in its infancy, initial attempts were not always successful. For instance, nearly half a century later when May ventured to perform the first rabbit to human corneal xenograft transplantation and failed after twenty-four unsuccessful attempts, his conclusion was that the unsuccessful outcome was due to "imperfect technique and the inability to keep the eyes properly bandaged" or as some surgeons would put it currently "due to an improper response by the eye" (8)**.** Finally in 1905, Zirm carried out the first successful transplantation thereby establishing keratoplasty as the oldest form of solid organ transplantation (9)**.** Over 100,000 keratoplasties are now performed annually - making corneal transplantation the most common form of organ transplantation worldwide (10)**.**

Several surgical sub-specialities make up modern keratoplasty (1). Lamellar keratoplasty involves the removal and replacement of tissue that is less than the total thickness of the cornea. Typically, lamellar grafts are relatively large (> 8 mm in diameter), and they replenish tissue excised by deep stromal dissection. As a rule, the host's Descemet's membrane and the endothelium are left undisturbed and provide the scaffold onto which the donor tissue can be laid. Lamellar keratoplasty is typically recommended for cases with anterior stromal pathologies and surface irregularities in which the endothelium is perfectly healthy. Penetrating keratoplasty,

which will be the subject of this dissertation, involves complete replacement of the corneal tissue. Indications for the procedure vary widely but ultimately aim to restore corneal morphology and function, and with it, visual acuity. Based on reports published by the Eye Bank Association of America (EBAA) in 2005, 20% of the >42, 000 keratoplasties performed that year were for patients with corneal edema resulting from prior cataract surgery (11). Other indications for penetrating keratoplasty are summarized in **Table 1**.

U.S. Eye Banks Reporting Indications for Corneal Transplants	2005
Post-cataract surgery edema	19.8%
Keratoconus	14.5%
Fuchs' Dystrophy	13.6%
Repeat Corneal Transplant	12.8%
Other degenerations or dystrophies	10.1%
Microbial changes	2.7%
Mechanical or chemical trauma	2.4%
Congenital opacities	1.1%
Post-refractive surgery	0.1%
Other causes of corneal opacification or distortion	22.8%
Total Corneal Transplants	48,474
Total Penetrating Keratoplasties	45,821

Table 1. Indications for Corneal Transplant in 2005 using EBAA Medical Advisory Board categories.

Corneal Allograft Rejection

Keratoplasty is undeniably the most common and successful form of solid organ transplantation in humans with at least 1 million transplants performed since 1961 (www.restoresight.org). Yet up to 20% of the keratoplasties performed each year in the United States undergo immune-mediated rejection (www.nei.nih.gov/health/cornealdisease) in spite of the use of topical corticosteroids, which by themselves carry the added risk of developing glaucoma and cataracts. Current epidemiological data posits that approximately 25% of transplanted corneas are expected to fail within 4-5 years (12).

Any of the three layers of the cornea are susceptible to immune mediated rejection. As mentioned previously, because the corneal epithelium turns over quite rapidly, destruction of this cell layer is typically of no consequence to the integrity of the whole graft. Nonetheless, immune responses restricted to the epithelium still immunize the host to the donor alloantigen and can escalate to full blown rejection. Stromal rejection is equally frequent, but in most hosts, does not lead to endothelial cell loss, which would irreversibly impair the tissue. The endothelial cell layer in humans has no replicative potential and is essential to maintaining corneal deturgescence. Immune-mediated insults to the endothelial cell layer lead to corneal swelling, collagen fiber disorientation, and eventual loss of corneal clarity due to edema. In patients with increasing corneal opacity, intensive treatment with topical steroids can reverse acute inflammation and minimize endothelial cell loss (13). In end-stage graft failure however, the endothelial cell density decreases to levels where control of corneal swelling and maintenance of corneal transparency is no longer sustainable.

Human studies examining the pathogenesis of rejection have been quite limited owing to the fact that prior to rejection, patients receive topical steroid treatment and therefore, might

experience altered forms of rejection with limited physiological relevance (14). Hence, most of our information into the pathogenesis of allograft rejection stems from animal models of transplantation. Although tremendously useful, it should be noted that the current models do not completely mirror the human process mainly due to the fact that rodent corneal endothelial cells are capable of division, and rodents themselves experience a more acute form of rejection (12). Nonetheless, the past 30 years of animal work has yielded invaluable insight into the process of allograft rejection.

Ocular Immune Reflex Arc in Corneal Allograft Rejection

The execution of allograft rejection can be conceptualized as being analogous to the motor reflex induced in response to a sensory stimulus (15). During the first phase of the immune response, the afferent phase, the resulting inflammation from organ transplantation activates professional antigen presenting cells (APCs), typically Langerhans cells (LCs) (16). Donor histocompatibility antigens are then processed by professional APCs, which subsequently transport the antigens to the regional lymphoid tissue as shown in **Fig. 2**. The histocompatibility antigens consist of the major histocompatibility complex (MHC) and minor histocompatibility (miH) antigens. The MHC is a gene complex that encodes a series of cell surface molecules while the miH genes encode intracellular antigenic peptides (17). Genetically identical individuals such as uniovular twins or syngeneic mice have identical histocompatibility antigens and can freely exchange grafts without the occurrence of immune-mediated rejection. Thus, the specificity of allograft rejection is genetically driven via histocompatibility antigen mismatches between donors and recipients. The activated antigen presenting cell population is thought to consist of a mixed bag of two subpopulations; donor cornea-derived passenger APCs and host-derived limbal and conjunctival APCs that engulf and process donor histocompatibility antigens

(18). The APCs home by way of the afferent lymphatics or blood vessels to the regional lymphoid tissue where they initiate antigen recognition and host sensitization.

Allosensitization consists of the second phase of the immune response and can be most likened to the sensory/relay neuron interaction in the reflex arc. Allosensitization activates the adaptive immune system and can take place through one of two independent pathways as shown in **Fig. 2** (19-21). In the direct pathway, host $CD4^+$ T helper cells expressing T cell receptors (TCRs) that can interact with donor APC MHC molecules are activated (18, 22). In contrast, indirect allorecognition requires presentation of processed MHC or minor antigens by host APCs (23). Depending on the cytokine signature of the APCs and the surrounding lymphoid microenvironment, the $CD4^+$ T cell can be polarized into one of several subsets and undergo oligoclonal expansion.

During the final phase of the alloresponse, the efferent phase, alloreactive $CD4^+$ T helper cells enter the general circulation from the lymphoid organs and migrate to the corneal tissue. Upon re-encountering alloantigens at the graft/host interface, the $CD4^+$ T cells initiate their allodestructive processes. This three-stage process termed the "ocular immune reflex arc" culminates in corneal allograft rejection.

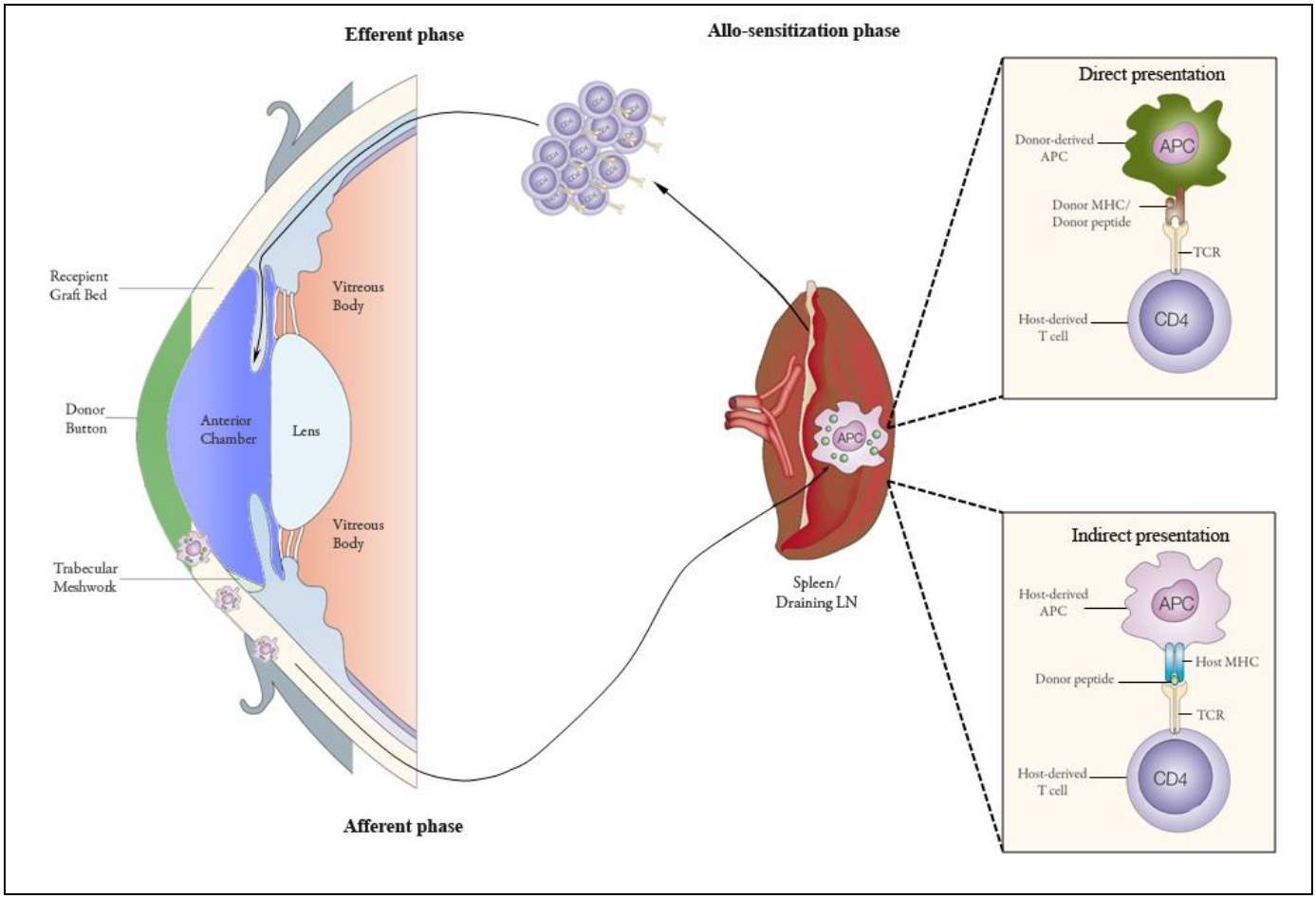

Figure 2. Ocular immune reflex arc. During the afferent phase of the immune response, donor cornea-derived APCs move to the regional lymph nodes via either the conjunctival lymphatics or aqueous outflow channels. Through the direct pathway of allorecognition, MHC molecules on the donor APCs present graft-derived antigens to host T cells, leading to the clonal expansion of $CD4^+$ T cells with TCR recognizing donor cornea MHC antigens. Alternatively, in the indirect route of allorecognition, recipient-derived APCs, originating from the limbus or conjunctiva, sensitize the host by presentation of donor-derived corneal peptides on host MHC to host T cells. During the efferent phase of the immune response, allospecific $CD4^+$ T cells egress from the iris and home back to the corneal button where they mediate their allodestructive responses.

$CD4^+$ T Cells in Corneal Allograft Rejection

$CD4^+$ T cells are critical to allograft destruction. Investigations on corneal allografts in rodents have revealed that *in vivo* depletion of $CD4^+$ T cells dramatically reduces the incidence of immune rejection (24-27). Additional evidence through adoptive transfer studies confirmed that the $CD4^+$ T cells can independently mediate corneal allograft rejection. Similarly, downregulation of $CD4^+$ T cell immune responses has been correlated with enhanced graft survival (28). Since its first association with allograft rejection, the $CD4^+$ T cell subset has expanded significantly into several discrete populations. Among the most extensively characterized T cell subsets are the Th1, Th2, Th17, and $CD4^+CD25^+$ T regulatory cell lineages.

T helper 1 (Th1) cells

Th1 cells are characterized by their activation of the T-bet transcription factor and their production of interferon-γ (IFN-γ), which can have multiple effects that stimulate macrophages and endothelial cells to produce proinflammatory factors (29, 30). The notion that Th1 cells were the primary mediators for corneal allograft rejection stemmed from observations in human keratoplasty patients and in rodent models of corneal transplantation, which noted the presence of $CD4^+$ T cells and IFN-γ in rejected corneal allografts (31, 32). Th1 cells mediate delayed-type hypersensitivity (DTH), which is closely associated with corneal allograft rejection, while maneuvers that downregulate donor-specific DTH correlate with long-term corneal allograft survival (27, 33-35). The precise effector mechanism by which the Th1 subset mediates graft rejection remains elusive. Possible candidates include soluble inflammatory mediators normally released by Th1 cells during DTH reactions. Indeed, increased protein and mRNA levels of TNF-α and IFN-γ have been detected in rejected corneas (32, 36). Also, corneal endothelial cells exposed to these cytokines undergo nitric oxide-induced apoptosis (37). By contrast, corneal

allograft rejection proceeds unimpeded in mice with deletion of genes encoding either TNF-α receptor 1 (TNFRI) or TNF-α receptor 2 (TNRII) thus highlighting redundancies in the rejection mechanisms (38). It has also been suggested that Th1-dependent allograft rejection might be mediated by the recruitment of accessory cells such as mononuclear cells. For instance, treatment of corneal cells with TNF-α and IFN-γ has been shown to upregulate expression of the cell adhesion molecules VCAM-1, ICAM-1, and E-selectin, which are required for recruiting mononuclear cells to the graft site (39-41).

T helper 2 (Th2) cells

Th2 cells express the GATA-3 transcription factor and elaborate IL-4, IL-5, and IL-13. In the classical T helper cell paradigm, $CD4^+$ Th2 cells cross-regulate Th1 cells and presumably suppress Th1-mediated immune responses (29). Accordingly, it has been suggested that skewing the Th1 alloimmune response to the Th2 pathway would enhance graft survival. One of the first hints that this was not the case stemmed from experiments in atopic diseases such as allergic conjunctivitis and allergic airway hyperreactivity (AHR), which are characterized by Th2-based immune responses. In these settings, the incidence and tempo of allograft rejection increase dramatically (42-44). Interestingly, in atopic hosts, allograft rejection appears to be mediated by a combination of allospecific Th1 and Th2 cells that act synergistically to exacerbate allograft rejection. It is interesting to note that only co-adoptive transfer of $Tim3^+$ $CD4^+$ T cells (Th1 cells) and $T1/ST2^+$ $CD4^+$ T cells (Th2 cells) to SCID mice was able to mirror the tempo and incidence of rejection observed in the atopic hosts (43). Thus, there are multiple pathways and mechanisms by which $CD4^+$ T cells can mediate corneal allograft rejection (**Table 2**).

The mechanisms used by Th2 cells to mediate corneal allograft rejection remain to be elucidated. However, recent observations suggest that Th2-mediated corneal allograft rejection

might occur through accessory cells that are activated by allospecific Th2 cells (45). Indeed, in allergic conjunctivitis-associated corneal allograft rejection in mice, significant infiltration of Th2 cells and eosinophils was noted at the rejection site (44). Moreover, eosinophils, the inflammatory cell population associated with Th2-based inflammation, have been detected in corneal allografts that have undergone rejection in humans with allergic conjunctivitis (46). It was thought that allospecific Th2 cells migrate to the graft site where they elaborate IL-5 and recruit eosinophils. Once at the tissue, the eosinophils might mediate damage to the corneal allograft through multiple effector mechanisms (47). Eosinophils secrete an array of cytotoxic granule cationic proteins such as major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN), which are capable of inducing tissue damage and dysfunction (48). Eosinophils can also release a variety of cytokines including IFN-γ and TNF-α, which can directly damage the corneal cells (49). Finally, eosinophils can serve as APCs that present antigen to T cells and cause mast cell degranulation, thereby amplifying the inflammatory response (50-52). Thus, eosinophils possess a plethora of mechanisms that could account for the exaggerated rejection of corneal allografts in hosts with allergic conjunctivitis. However, two observations indicate that eosinophils are not necessary, and probably not even involved in corneal allograft rejection in hosts with a Th2-biased alloimmune responses. The first observation is based on a simple experiment in which allergic conjunctivitis was induced in one eye and a corneal allograft was placed onto the contralateral eye that was not challenged with allergen and did not manifest allergic conjunctivitis (44). Although the eye that was challenged with allergen expressed allergic conjunctivitis and contained abundant numbers of eosinophils, the contralateral eye was free of eosinophils, yet the tempo and incidence of corneal allograft rejection in the non-allergic eye replicated the

exacerbated rejection that occurred when corneal allografts were placed onto eyes with active allergic conjunctivitis (44). Thus, the most plausible explanation for the exacerbation of corneal allograft rejection in this setting suggests that allergic conjunctivitis produces a systemic effect that denies the corneal allograft its immune privilege. A second observation supporting the hypothesis that allergic diseases exacerbate corneal allograft rejection by exerting a systemic effect stems from studies using a mouse model of airway hyperreactivity, which is a model of allergic asthma. Similar to mice with allergic conjunctivitis, mice with short ragweed (SRW) pollen-induced AHR experienced a dramatic increase in the tempo and incidence of corneal allograft rejection compared to non-allergic hosts (42). Thus, allergic disease, even in an organ distant from the eye can exacerbate corneal allograft rejection leading to the inescapable conclusion that allergic diseases exert a systemic effect that abolishes immune privilege of corneal allografts.

T helper 17 (Th17) cells

Recently, a newly identified $CD4^+$ Th17 helper cell subset has blurred the distinction between Th1 and Th2 cell-mediated inflammation (53, 54). Th17 cells have been described based on the secretion of the cytokine IL-17A and the expression of the transcription factor RORγt (53-55). Functionally, the Th17 cell subset is important for the clearance of several pathogens that are not adequately handled by the Th1 and Th2 lineages.

The Th17 cell effector cytokine, IL-17A, regulates several key inflammatory molecules that activate the innate immune response and modulate neutrophil homeostasis (56-60). Several reports have linked Th17 cells to the pathogenesis of a number of autoimmune diseases, which were previously thought to be Th1-mediated (61-64). With respect to transplantation rejection, several experimental models have reported a pathogenic role for this subset in allograft rejection

(65, 66). The initial observations indicating an adverse relationship between the outcome of transplantation and the Th17 subset came from work in a mouse cardiac allograft model demonstrating an enhancement in graft survival following blockade of IL-17A (67, 68). Additional work performed in a murine corneal transplantation model showed that IL-17 KO hosts experienced a decrease in the rate but not the incidence of allograft rejection (69). Adoptive transfer studies aimed at directly linking the Th17 subset to graft rejection yielded mixed results with the adoptively transferred Th17 cells converting to the Th1 lineage *in vivo* (70). To date, the majority of reports linking the Th17 subset to graft rejection are predominantly based on association, with very limited to non-existent functional evidence available. Since multiple cells produce IL-17 and redundant pathways can effect rejection, functional validation for a role of Th17 cells in graft rejection has been hampered. Moreover, no significant correlation between IL-17A and transplant rejection has been found in the clinical setting (71). On the other hand, IL-17A has also been shown to have several regulatory and protective effects. In mouse models of colitis and graft-versus-host disease, IL-17A has been shown to mitigate the severity of the disease by limiting Th1-associated inflammation (72-74). Additionally, IL-17A has been shown to negatively regulate asthma via inhibition of dendritic cells and chemokine synthesis in sensitized hosts (75). Thus, our understanding of Th17's role in allograft rejection is limited.

$CD4^+CD25^+$ T regulatory cells

$CD4^+CD25^+$ regulatory T cells (Tregs) are associated with various forms of immunologic tolerance including the immune privilege of corneal allografts. $CD4^+CD25^+$ Tregs promote allograft survival in mice and have been implicated in the success of organ allografts in clinical settings (76-79). $CD4^+CD25^+$ Tregs make up 5-10% of the $CD4^+$ T cell population and are identified by the expression of their signature transcription factor, Foxp3 (80, 81). $CD4^+CD25^+$

Tregs are involved in a wide range of activities that maintain immunological homeostasis and prevent autoimmune diseases (82-84). $CD4^+CD25^+$ Tregs can come in two basic varieties: natural Tregs and induced Tregs. Natural Tregs display a T cell receptor (TCR) repertoire that is specific for self antigens and block autoimmune responses. By contrast, induced Tregs are generated in response to specific antigens and utilize a limited TCR repertoire and, as such, have been implicated in alloantigen-specific tolerance (81). In the context of corneal transplantation, $CD4^+CD25^+$ Tregs are induced during keratoplasty and have a profound influence on the fate of corneal allografts (85). Interestingly, the survival of corneal allografts is closely correlated with the level of Foxp3 expression in $CD4^+CD25^+$ Tregs and not on the number of $CD4^+CD25^+$ Tregs, as has been reported with other categories of organ allografts (86-88). $CD4^+CD25^+$ Tregs promote graft survival via their production of two well-known soluble immunosuppressive molecules, transforming growth factor-β1 (TGF-β1) and interleukin-10 (IL-10), which directly inhibit T cell proliferation(89, 90). Although not described in the corneal transplantation setting, membrane-bound suppressive mediators have also been reported to be used by Tregs and include molecules such as cytotoxic T-lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR), and cell surface tethered-TGF-β1 (81, 84).

Mechanistically, interaction between CTLA-4 derived from $CD4^+CD25^+$ Tregs and CD80/86 present on dendritic cells is thought to upregulate the expression of indoleamine 2,3-dioxygenase (IDO) (91). IDO catabolizes the amino acid tryptophan, which is essential for $CD4^+$ T cell survival. In addition to starving the T cells, L-kynurenine, a tryptophan metabolite, can render effector T cells apoptotic. The role of IDO in promoting corneal allograft survival is unresolved. It has been reported that human corneal cells express IDO mRNA and protein, and inhibit the proliferation of T cells (92). In mice, IDO gene transfer to corneal allografts results in

a significant prolongation of corneal allograft survival in a donor/host combination with an exceptionally high incidence of rejection (93). However, it is not known if IDO is generated directly or indirectly by Tregs or the degree to which it influences corneal allograft survival. It is possible that Tregs acting *in situ* might generate IDO or induce corneal cells to produce IDO, which would disable effector T cells that infiltrate the corneal allograft and thereby promote graft survival. GITR is the third membrane-bound molecule that is employed by $CD4^+CD25^+$ Tregs to mediate suppression. The dynamics of $CD4^+CD25^+$ Tregs and GITR ligand (GITRL) interaction that results in alloimmune suppression is currently still under investigation. $CD4^+CD25^+$ Tregs are known to constitutively express high levels of GITR (94, 95). The current paradigm is that during an inflammatory response, $CD4^+CD25^+$ Tregs expressing GITR interact with GITRL expressed on APCs. This interaction, in conjunction with IL-2 produced by effector cells, leads to an expansion of the $CD4^+CD25^+$ Tregs which subsequently suppress the effector T cell population (96).

CD4+ T cells	Th1	Th2	Th17	Treg
Effector Cytokine	IFNγ, TNFα, IL-2	IL-4, IL-5, IL-13	IL-17A,IL-17F, IL-21, IL-22	IL-10, TGF-β1
Obligatory Transcription Factor	T-bet	GATA3	RORγT	Foxp3
Effector Function in Transplantation	Classical mediator of allograft rejection	Exacerbate allograft rejection in atopic hosts	Novel mediator of allograft rejection	Promote allograft survival
Possible Effector Mechanisms	• Direct damage to the cornea via IFNγ and TNFα • Upregulation of adhesion molecules and chemokines • Complement fixing alloantibodies • Contact-dependent cytolytic activity via Fas-L and perforin	• Intensification of immune response via a Th1 and Th2 pathway • Inhibition of T regulatory cells • Eosinophil derived cytotoxic granules cationic proteins and cytokines • IgE-mediated eosinophil and mast cell degranulation		• Soluble molecules such as IL-10 and TGFβ1 suppress effector cells • Contact - dependent direct immune suppression via membrane TGF-β1 • Immune modulation of antigen presenting cells by CTLA-4 • Antigen non-specific expansion via GITR ligand interaction

Table 2. The role of $CD4^+$ T cells in keratoplasty.

Immune Privilege of the Cornea

Compared to other forms of solid organ transplantation, which require systemic immunosuppressive treatment and HLA typing, corneal transplantation commonly enjoys a success rate of up to 90% while relying only on the use of topical corticosteroids as the sole immunosuppressive modality to prevent immune rejection (97)**.** This success is even more remarkable when one considers that keratoplasty occurs in the absence of HLA histocompatibility matching. Prospective studies in animal models have shown that in the absence of immunosuppressive agents, only 50% of fully allogeneic corneal allografts (i.e., mismatches at the entire MHC plus the full array of minor histocompatibility alleles) undergo immune rejection compared to a 100% incidence of rejection for skin grafts involving the same donor/host rodent strains (98-100)**.** These apparent violations of the laws of transplantation are the basis for the notion that corneal allografts are endowed with "immune privilege".

Seminal work demonstrating the unusual immunological properties of the anterior chamber of the eye can be traced to Van Dooremaal. In 1873, while developing an animal model for the study of cataractogenesis, Van Dooremaal reported the successful transplantation of allogeneic and xenogeneic tumor tissues and/or fetal cartilage into the AC of rabbit eyes (101)**.** Follow up studies by Greene in the 1940's similarly described the remarkable survival of human tumors in the eye and their progressive growth in the AC and brain of guinea pigs, rabbits, goats, sheep, and rats (102-104). Nonetheless, initial dissection of the physiological basis of this ocular phenomenon had to await investigations by Sir Peter Medawar, the father of transplantation. After observing the striking survival of orthotopic corneal allografts transplanted to the ocular surface and heterotopic skin allografts placed into the AC of the rabbit eye, Medawar recognized the unique immunological properties that the cornea and the anterior segment were endowed

with and coined the term “immune privilege” (105). Medawar proposed that the underlying condition leading to immune privilege was immunological ignorance. Specifically, he concluded that the absence of lymphatic drainage in the eye and thus, the sequestration of the non-self antigen within the AC - isolated from the systemic immune system - prevented immunological rejection (106, 107). Evidence that a breach in the isolated ocular compartment via increased vascularization of the graft bed led to immune-mediated rejection of ocular implants further supported Medawar’s hypothesis (107, 108).

Appealing in its simplicity, the immunological ignorance explanation, however, came under fire with the observation that corneal allografts placed in the anterior chamber did in fact elicit an alloimmune response (109). Initial reports described the systemic emigration of alloantigen from the ocular environment (109-111). Observations by Streilein and Kaplan further challenged the hypothesis by demonstrating that semi-allogeneic (F1) lymphoid cells injected intracamerally (IC) led to significant production of hemagglutinating antibodies against donor histocompatibility antigens. Interestingly, while IC-injected alloantigens activated humoral immunity, the cell-mediated component of the immune response appeared to be stifled as evidenced by the significant delay in the rejection of orthotopic donor-specific skin allografts in the IC-primed hosts (112-114). These observations provided ample support to refute the hypothesis that immune privilege in the eye was the result of immunological ignorance.

The high turnover rate of the corneal epithelial cell layer has also been put forward as a possible mechanism via which the corneal tissue becomes non-immunogenic. Indeed, in mouse models of keratoplasty, as early as 15 days post transplantation, the donor epithelium was shown to be totally replaced by the recipient epithelial cells (115). However, similar to the avascularity hypothesis, clinical observations and animal studies have provided compelling evidence to the contrary. For instance, patients can reject their grafts years after the initial keratoplasty while

immune rejection in mice with long term surviving corneal allografts can be accelerated by immunizing the recipients with donor antigens (100). Additional animal studies using chromatin sex markers and radiolabels as trackers for donor corneal cells have revealed that the latter persist in surviving corneal allografts (116, 117).

Thus far, several organs and tissues have been recognized for their varying degrees of immune privilege. In addition to the cornea and the anterior chamber of the eye, other sites include the brain, the hamster cheek pouch, hair follicles, testes, and the pregnant uterus (4). Studies into these immune privileged sites, particularly the CNS, have further repudiated the immunological ignorance explanation (118, 119). Instead, extensive investigation by Streilein and colleagues for the past 30 years has revealed that immune privilege is the product of multiple anatomical, physiological, and immunoregulatory processes (4, 99). Studies in rodent models of keratoplasty have shown that immune privilege of corneal allografts is attributed to three conditions: a) intrinsic features of the corneal physiology that make the tissue imperceptible to the immune system; b) ocular immunoregulatory mechanisms that induce tolerogenic immune responses to antigens placed within the eye; and c) efferent blockade of the immune reflex arm. The following sections will focus on evolutionary adaptations of immune privilege that serve to protect the delicate corneal tissue that is essential for vision.

Afferent Phase Blockade

Several intrinsic features of corneal physiology make the tissue imperceptible to the immune system. Among them, the avascularity of the corneal graft bed limits antigen sampling at the graft/host interface, thereby hampering the afferent phase of the ocular immune reflex arc. Nonetheless, in spite of being necessary to the maintenance of corneal immune privilege, graft bed avascularity is not sufficient. As mentioned above, direct allorecognition is one of the pathways via which allosensitization is thought to proceed. Other tissues such as the skin contain

a dense network of LCs, which serve as potent activators of the immune cascade leading to graft rejection. Interestingly, the anatomical placement and physiology of LCs cells present on the corneal tissue differs significantly when compared to the skin. First, MHC class II expressing LCs appear to be absent from the central corneal epithelium, thereby limiting passenger APCs transfer and stifling subsequent direct allorecognition. In contrast, trauma to the central cornea and subsequent migration of MHC class II-positive LCs from the periphery of the cornea into the donor corneal button significantly compromises graft survival (120). Thus, the local distribution of LCs appears to be critical to corneal immune privilege. Recent reports showing constitutive presence of immature MHC class II negative LCs in the central cornea suggest that the maturity status of corneal LC might be as important as their location. Nevertheless, restricting systemic alloantigen transfer by either limiting lymphatic drainage from donor tissue, and recipient APCs access to the allograft or strategically making the central cornea devoid of mature passenger LCs significantly impairs the afferent phase of the ocular immune reflex arc.

Allosensitization Phase Blockade

If the strategies to block the afferent phase of the immune response prove not to be sufficient, additional regulatory mechanisms put in place by evolution are set into motion to prevent irreparable damage to the cornea. As one of the most elegant and complex outcomes of evolution, anterior chamber-associated immune deviation (ACAID) has elicited much attention notably for its ability to enhance corneal allograft survival in murine models of keratoplasty.

Initial observations hinting at ACAID were made in the 1970s when it was discovered that IC-injected alloantigenic cells were not only perceived by the systemic immune apparatus, but in fact elicited a systemic downregulation of alloimmune responses (112, 114). Further investigations revealed that the phenomenon specifically targeted the cell-mediated component

of the immune response as exemplified by the significant delay in the rejection of orthotopic donor-specific skin allografts from the same donor strain used for the IC immunizations. Initially termed "lymphocyte-induced immune deviation" (LIID) (112, 114) , subsequent studies by Niederkorn and Streilein highlighted the critical requirement of the AC compartment to the induction of immune deviation, thereby establishing that ACAID was not characteristic of a unique lymphoid population as previously suggested (112, 114). Hence, the term anterior chamber-associated immune deviation (ACAID) emphasized the requirement for the AC in the generation of this form of systemic, antigen-specific suppression of cell-mediated immune responses (121).

ACAID can be induced by AC injections of antigen or by corneal transplantation through the sloughing of corneal cells into the AC. A variety of antigens have been assessed in terms of their ability to induce ACAID including viral proteins (122), soluble proteins (123), and histocompatibility antigens (112). So far, all soluble antigens tested were shown to elicit ACAID. The deviation mediated by ACAID selectively leads to the antigen-specific suppression of Th1-based immunity while leaving the induction of cytotoxic T lymphocyte (CTL) responses unhampered. CTL induced in ACAID are primed, clonally expanded, but do not fully differentiate (124-126). The humoral response induced by ACAID also has a unique signature. Introduction of antigen into the AC modulates antibody production from complement-fixing isotypes to the synthesis of non-complement fixing antibodies (IgG1) (127, 128).

The last thirty years have led to an extensive elucidation of the mechanistic basis of the phenomenon. It has been demonstrated that within 48 hrs post antigen injection or alloantigen shedding into the AC following corneal transplantation, unique, tolerance-inducing $F4/80^+$ APCs are activated and migrate from the iris/ciliary body into the AC. The alloantigen-loaded $F4/80^+$

APC population exits the AC via the trabecular meshwork into the canal of Schlemm and eventually enters the host venous circulation. Once blood-borne, the ocular $F4/80^+$ APCs first home to the thymus where they generate $CD4^-CD8^-NK1.1^+$ thymocytes. The $CD4^-CD8^-NK1.1^+$ population then migrates to the spleen where together with $F4/80^+$ ocular APCs, B cells, NKT cells, and γδT cells participate in the generation of splenic suppressor cells. These ACAID suppressor cells have been characterized extensively and consist of a $CD4^+$ afferent suppressor cell population, and $CD8^+$ efferent suppressor cells. The $CD4^+$ afferent suppressor cells inhibit the primary proliferative responses to alloantigens expressed by the corneal cell layers, thereby suppressing clonal expansion of Th1 inflammatory lymphocytes. In agreement to prevailing paradigms, it was initially proposed that ACAID-induced tolerance arose from a deviation of the alloimmune response from a Th1 to a Th2-based response. Corroborating evidence for this hypothesis came from initial studies demonstrating that spleens from animals expressing ACAID showed decreased IL-2 and IFN-γ production, but increased IL-10 and IL-4 production (129). However, subsequent studies demonstrated that IL-10, not IL-4, is the critical cytokine involved in ACAID (123, 129, 130). Further investigation demonstrated that IL-10 is required for the generation of Tregs and their effector functions. IL-10 derived from ACAID APCs is vital for the induction of Tregs, which in turn, require IL-10 to mediate their regulatory functions (123, 131). Excluding its requirement for IL-10, the cytokine milieu requirements, molecular mechanisms, and kinetics of ACAID $CD4^+CD25^+$ Treg-mediated suppression are not well understood.

The success of a corneal allograft and thus, the tissue's immune-privileged status are linked to ACAID. Several experimental protocols that abolish ACAID provide indirect evidence to that inference (34, 35, 99, 132, 133). Of interest, splenectomy and systemic depletion of IL-10 have been shown to abolish ACAID and to significantly exacerbate the incidence of allograft

rejection (123). Similarly, in addition to abrogating ACAID, depletion of NKT and γδ T cells by means of genetic manipulation or antibody treatment dramatically increases the severity of allograft rejection (134, 135). These observations, in addition to the fact that induction of ACAID prior to transplantation significantly improves graft success, provide compelling evidence to the immunoregulatory role of ACAID in the allosensitization phase of the ocular immune reflex arc.

Efferent Phase Blockade

Once the host's $CD4^+$ T cells have been primed, there are additional layers of protection that stand between the immune cells and the allograft. The first layer of protection involves ACAID $CD8^+$ Tregs. Generated by ACAID $CD4^+$ Tregs in an IL-10-dependent mechanism, the ACAID $CD8^+$ regulatory cells are unique in their ability to inhibit the expression of cell-mediated immune responses upon a second exposure to the antigen. Characterization of this $CD8^+$ population has yielded only limited insight into its mode of action. Nonetheless, available data suggests that the activation of the population is IFN-γ-dependent and that the $CD8^+$ Tregs produce significant levels of TGF-β (136). Yet, further investigation revealed that the $CD8^+$ Treg-mediated suppression is FasL- and TGF-β2-independent (137, 138), indicating the existence of either redundant or novel regulatory mechanisms.

The next protective layer is made up of soluble molecules within the AH, which bathes the corneal endothelium. The AH contains a plethora of immunosuppressive molecules that prevent immune cell activation and inhibit production of inflammatory mediators (4). Some of the molecules include TGF-β, α-melanocyte-stimulating hormone (α-MSH), vasoactive intestinal peptide (VIP), calcitonin gene-related protein (CGRP), macrophage migration inhibitory factor (MIF), soluble FasL (sFasL), complement regulatory proteins (CRPs), IDO, somatostatin (SOM)

and IL-1 receptor antagonist (IL-1Ra) (139-144). These factors contribute to immune privilege in a variety of ways, including, but not limited to, the inhibition of DTH (145, 146), the inhibition of NK cell-mediated cytolysis (140, 147), the suppression of T cell proliferation (142-144, 148), the suppression of complement activation (149), the suppression of nitric oxide generation by macrophages (141), and evasion of apoptosis (150, 151).

The final layer of protection is intrinsic to the corneal tissue itself and relates to the expression pattern of particular surface molecules. During corneal allograft rejection, allospecific T cells and neutrophils egressing from the iris vasculature target the donor corneal endothelium for destruction. In order to limit the local ocular inflammation that might lead to blindness, corneal endothelial cells express several death ligands notably, FasL, TRAIL, and PD-L1 which induce apoptosis of the infiltrating leukocytes. Expressed abundantly on the corneal cell layers, these molecules effectively eliminate pathogenic lymphocytes without provoking inflammation or tissue necrosis in the eye (152-158). This elegant component of corneal immune privilege has been shown to contribute significantly to corneal allograft success. For instance, BALB/c mice receiving orthotopic corneal transplants from C57BL/6-gld/gld (generalized lymphoproliferative disease) mice, which lack functional FasL experience significantly higher rates of graft rejection when compared to WT C57BL/6 donors (155, 159). Subsequent studies with mice lacking PD-L1 further confirmed the crucial need of these death ligands for corneal immune privilege and successful corneal allograft survival.

In addition to its apoptosis inducing capabilities, the endothelial cell layer can evade destruction by suppressing the infiltrating lymphocyte population. Surface expression of TGF-β is one means by which the endothelial cell layer can directly anergize the effector immune cells (160). In addition to direct immunosuppression, the corneal endothelial cell layer in mice

expresses GITRL, which allows for local expansion of the $CD4^+CD25^+$ Tregs, which can subsequently suppress incoming pathogenic lymphocytes (161). The molecular strategies evolved by the cornea also serve to regulate the innate immune system, particularly, the complement system. Activation of the complement cascade leads to the deposition of membrane attack complexes on the surface of corneal cells and their subsequent osmotic lysis. Byproducts of the membrane attack complex (MAC) expand the inflammatory response by further luring immune cells. To protect the graft against unbridled complement activation, CRPs are expressed in the AH and throughout the ocular environment, and are predominantly displayed on the corneal epithelial cells (149, 162). Administration of neutralizing antibody against CRPs results in extensive intraocular inflammation and damage to ocular tissues (163).

The corneal tissue is also endowed with several features that drastically reduce its immunogenicity. Firstly, corneal cells do not constitutively express MHC class II antigen. In addition, MHC class Ia expression is significantly reduced, especially on corneal endothelial cells when compared to cells from non-immune-privileged sites (164, 165). The limited expression of MHC class I molecules protects the corneal tissue from class I-restricted CTL-mediated insults (4). However, limited MHC class Ia expression by the corneal endothelial cells also has its downside, making the endothelial cell layer more susceptible to NK cell-mediated destruction. As a compensatory mechanism, corneal endothelial cells have increased expression of non-classical class Ib MHC molecules, which serve as inhibitory signals to NK cells, thereby protecting the class Ia negative cells. As mentioned previously and although not intrinsic to the tissue itself, normal corneas lack MHC-class-II expressing APCs that would typically activate alloreactive effector T cells *in situ* (120, 166).

Molecule	Effect on Immune System
TGF-β	Suppresses activation of T cells; promotes immune deviation ; down-regulates MHC class I expression on corneal cells; suppresses NK cells
VIP	Inhibits T cell activation and proliferation; inhibits DTH
CGRP	Inhibits production of proinflammatory factors by macrophages
MIF	Inhibits NK cells
sFasL	Suppresses neutrophil recruitment and activation
CRP	Disables complement cascade
IDO	Depletes tryptophan and “starves” T lymphocytes
α-MSH	Inhibits DTH and production of proinflammatory factors by macrophages
SOM	Suppresses IFN-γ production by activated T cells; induces production of α-MSH
IL-1Ra	Regulates IL-1-induced corneal inflammation

Table 3. Immunosuppressive and Immunoregulatory Molecules in the Aqueous Humor. TGF-β, transforming growth factor beta; VIP, vasoactive intestinal peptide; CGRP, calcitonin gene-related peptide; MIF, macrophage migration inhibitory factor; FasL, Fas ligand; CRP, complement regulatory proteins; IDO, indoleamine 2,3-dioxygenase; α-MSH, alpha-melanocyte stimulating hormone; SOM, somatostatin.

Molecule	Effect on Immune System
FasL	Induces apoptosis of activated T cells and neutrophils
TRAIL	Induces apoptosis of macrophages and neutrophils
PD-L1	Induces apoptosis of activated T cells
MHC class 1b	Inhibits NK cell and CTL activity
CRP	Inhibits complement cascade
mTGFβ	Suppresses activation of T cells; promotes immune deviation ; down-regulates MHC class I expression on corneal cells; suppresses NK cells
GITRL	Causes local expansion of T regulatory cells

Table 4. Immunosuppressive and Immunoregulatory Molecules Expressed by the Cornea. FasL, Fas ligand; TRAIL, Tumor necrosis factor related-apoptosis inducing ligand; PD-L1, Programmed cell death ligand-1; CRP, complement regulatory proteins; mTGF-β, membrane transforming growth factor beta; GITRL, glucocorticoid-induced tumor necrosis factor receptor family-related gene ligand.

Apoptosis and Corneal Immune Privilege

Apoptosis is a genetically encoded program used by living cells to initiate self-destruction as part of normal development and maintenance of homeostatsis (167-170). Because this process limits inflammation and bystander cell damage, it is the pathway of choice for maintaining corneal immune privilege. Apoptosis can be triggered by a wide variety of stimuli and is dependent on the proteolytic activity of caspase proteases. The cleavage of numerous proteins by the caspases leads to phagocytic recognition and engulfment of the dying cells.

At the molecular level, apoptosis can proceed via either the extrinsic pathway or intrinsic pathway (170). In the extrinsic pathway, engagement of death ligands such as FasL to their cognate receptors activates caspases which initiate the apoptotic process. In contrast, in the intrinsic pathway, mitochondrial outer membrane permeabilization (MOMP) is the initial trigger that leads to caspase activation through the release of cytocrome C. Although the precise mechanism has not been worked out, it is speculated that the balance between pro- and anti-apoptotic members of the BCL-2 family of proteins modulates MOMP. Work here will focus on three members of the BCL-2 family, notably BCL-2, BCL-xL, and BAX. BAX is a pro-apoptotic member of the family and is thought to induce apoptosis by disrupting mitochondrial integrity. BCL-2 and BCL-xL make up the anti-apoptotic members and function by inhibiting BAX. In healthy cells, all members of the BCL-2 family of proteins are expressed constitutively. However, following specific trigger events, the balance of expression is skewed towards the pro-apoptotic members thereby leading to apoptosis.

In the establishment of corneal immune privilege, both the extrinsic and intrinsic pathways are involved. As mentioned previously, both corneal cells and the AH express FasL, and can therefore both activate the extrinsic apoptotic pathway (155). Indeed, ligation of FasL to

its death receptors expressed by infiltrating lymphocytes leads to the apoptosis of the immune cells in the anterior chamber. In contrast, factors present within the AH can also hamper the instrinsic pathway to protect the corneal endothelial cells from programmed cell death. For instance, the iris/ciliary body cells have been shown to secrete a protein or proteins in the AH that upregulates corneal endothelial cell expression of BCL-2 and inhibits their apoptosis (151). Thus, by careful regulation and localized expression of pro and anti-apoptotic molecules, corneal immune privilege is established.

Current Investigations

As noted earlier, corneal allografts enjoy an immune privilege that is unrivaled in the field of transplantation. To investigate this exceptional form of tolerance, our laboratory uses a well-characterized mouse model of corneal transplantation in which donor and recipient are mismatched at both major and minor histocompatibility loci. 50% of the allografts enjoy indefinite survival while the rest are rejected via the Th1 lineage. The current paradigm has, however, been challenged recently based on the observation that IFN-γ-deficient mice experience a higher incidence of graft rejection than mice with a normal IFN-γ repertoire (171). The Th17 cell subset is a recently described helper T cell subset that has been linked to the pathogenesis of several autoimmune diseases classically thought to be Th1-mediated (61-64). We thus tested the hypothesis that Th17 cells mediate corneal allograft rejection by depleting its effector cytokine, IL-17A. Unexpectedly, the incidence of rejection increased to 90%.

For the first specific aim of this study, the pathogenesis behind the exacerbated allograft rejection associated with IL-17A depletion was investigated. Previous reports suggested that a cross-regulation was present between Th1 and Th17 cells (53, 72). In mouse models of colitis and graft-versus-host disease, IL-17A has been shown to mitigate the severity of the disease by

limiting Th1-associated inflammation (72, 73). Experiments were conducted to examine the loss of cross-regulation hypothesis. Our observations uncovered a novel cellular mediator of allograft rejection whose functional ability to mediate graft rejection was assessed.

The observation that depletion of IL-17A exacerbated allograft rejection led us to the second specific aim of this study, which examined the requirement of IL-17A for corneal immune privilege. The role of this cytokine in modulating local immunosuppression and apoptosis at the level of the cornea was initially assessed. Additionally, we also examined the possible interplay between $CD4^+CD25^+$ T regulatory cells and IL-17A in maintaining corneal allograft survival. Several reports have identified IL-17A-producing $CD4^+CD25^+$ Tregs (172, 173). In the corneal transplantation setting, the cytokine milieu requirements, molecular mechanisms, and kinetics of Treg-mediated suppression are not well understood. The current proposal investigated the IL-17A requirement for the $CD4^+CD25^+$ Treg-mediated suppression and further characterized the mechanisms by which this regulatory population establishes corneal immune privilege.

Since orthotopic corneal allografts are placed directly over the AC of the eye, it has been proposed that the sloughing or shedding of corneal alloantigens into the AC would induce ACAID and promote allograft survival (35, 174). Accordingly, the final section of this dissertation investigated a requirement for IL-17A by ACAID. Contrary to our initial hypothesis which considered corneal immune privilege and ACAID to be one in the same, it appears that the two are distinct phenomena with unique molecular and cellular attributes.

CHAPTER TWO

MATERIALS AND METHODS

Animals

Eight to ten week-old female mice were used in all experiments. BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were purchased from Taconic Farms (Germantown, NY) and BALB/c nude mice were obtained from the National Cancer Institute (Frederick, MD). STAT6 knockout (KO) mice were purchased from the Jackson Laboratories (Bar Harbor, ME). C3H/HeJCr (H-2^k) mice and BALB/c nude mice were obtained from the National Cancer Institute (Frederick, MD). All experimental animals were treated in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research.

Orthotopic corneal allograft and clinical evaluation of grafted corneas

Balb/c mice were given orthotopic corneal grafts from C57BL/6 donors on the right eye and were scored twice a week for opacity, neovascularization and edema as described previously (175). Briefly, degree of opacification ranged between 0 to 4+; with 0 = clear; 1+ = minimal superficial opacity; 2+ = mild deep stromal opacity with pupil margin and iris visible; 3+ = moderate stromal opacity with pupil margin visible, but iris structure obscured; and 4+ = complete opacity, with pupil and iris totally obscured. Corneal grafts were considered rejected upon two successive scores of 3+.

Cells

C57BL/6 mouse epithelial cell lines were previously generated by transformation with human papilloma virus *E6/E7* genes (151). These cells were maintained in complete minimal essential medium (MEM; JRH Biosciences, Lenexa, KS) containing 1% L-glutamine (Biowhittaker,

Walkersville, MD), 1% penicillin, streptomycin, fungizone (Biowhittaker), 1% sodium pyruvate (Biowhittaker), 10% fetal calf serum (Hyclone Labs), and 500 mg/ml geneticin (Gibco- BRL).

Animal Treatments

Anti-IFN-γ hybridoma (catalog number HB170; R4-6A2) and anti-CD25 hybridoma (catalog number TIB-222; PC615.3) were purchased from American Type Culture Collection. Rat anti-mouse CD8 monoclonal antibody was purified from the YTS 169.4 hybridoma. Anti-IL-17A monoclonal and polyclonal antibodies were produced by the UT Southwestern Hybridoma Facility as described previously (64). Monoclonal antibodies were isolated from hybridoma cultures and affinity purified. Rat IgG was purchased from Sigma-Aldrich. BALB/c mice were injected I.P. with 500μg of antibody daily from day -4 to day -2 and corneal transplants were grafted on day 0. Biweekly injections of the antibodies were continued up to day 60. For treatments with recombinant mouse IL-17A (rmIL-17A), animals were given either 0.3 or 0.5μg of rmIL-17A on day -2 and every other day onwards. For cyclophosphamide experiments, mice were injected IP with cyclophosphamide (Sigma-Aldrich) at a dose of 100 mg/kg the day before AC alloantigen or orthotopic corneal transplantation and at 7 day intervals thereafter (176).

DTH Assay

An ear swelling assay was used to measure DTH responses to C57BL/6 alloantigens as described previously (177). A cell suspension of 4×10^6 mitomycin-C-treated C57BL/6 splenocytes in 20 μl of Hanks' balanced salt solution (HBSS) was injected into the right-ear pinna of BALB/c mice. The left-ear pinna received 20 μl of HBSS without cells and served as a negative control. Results were expressed as: specific ear swelling = (24-h measurement – 0-h measurement) for experimental ear – (24-h measurement – 0-h measurement) for negative control ear.

Preparation of Antigen Presenting Cells

APCs were isolated from spleen cells of naive C57BL/6 or C3H mice. Briefly, cells were incubated with NH_4Cl erythrocyte lysis solution, washed, and resuspended at $2x10^6$ cells/ml of HBSS with 400 μg/ml mitomycin-C. The cell suspension was incubated at 37°C for 1 hr and washed 3X with HBSS and was used as a source of APCs in indirect mixed lymphocyte reactions (MLR). For the indirect MLR, C57BL/6 cell lysate was initially generated by resuspending C57BL/6 splenocytes at $3x10^7$ cells per ml of HBSS and sonicating the suspension with 10 x 1 second pulsations. Lysates were frozen at -80ºC for 10 mins and thawed at 37ºC for 5 mins for two cycles. BALB/c APCs were isolated by incubating the cell suspension of splenocytes in two 100-mm Primaria plates (5 ml each plate) at 37°C for 1 hr. Non-adherent cells were removed by vigorous washing. Adherent APCs were cultured in a 100-mm Primaria plate containing 4 ml complete RPMI medium supplemented with 10% FBS and pulsed with the C57BL/6 cell lysate (1 ml). Cell cultures were incubated at 37ºC overnight.

Mixed Lymphocyte Reactions

Spleen cells were harvested from BALB/c mice 4–7 days after rejection of the C57BL/6 corneal allografts or at 3 weeks post transplantation in acceptors. $CD4^+$ T cells were enriched by positive selection using rat anti-mouse CD4-conjugated magnetic microbeads (Miltenyi Biotec Inc.). Purified $CD4^+$ T cells were incubated at $1x10^6$ cells per well with respective APC populations at a 1:1 ratio for 5 days at 37ºC in 2 mls of complete RPMI. ELISAs for IL-4, IL-5, IL-13, IL-17A, IFN-γ, and TNF-α were performed on culture supernatants according to manufacturer's instructions (R&D Systems).

Immunohistochemistry

Eyes from corneal allograft rejector mice were enucleated after two successive scores of 3+ and fixed in 10% formalin and were processed for histology. Sections (4-µm) of paraffin-embedded tissue were labeled with mAb against T1/ST2 (DJ8; MD Biosciences) to detect Th2 cells using the Vectastain Elite ABC system (Vector Laboratories). Antibody specificity was validated using a rat IgG1 isotype control (BD Pharmingen/eBioscience).

Adoptive Transfer of Th2 Cells

Spleen cell suspensions were obtained from anti-IL-17A-treated BALB/c recipients 4-7 days after rejection of C57BL/6 corneal allografts. $CD4^+$ T cell enrichment was carried out using the magnetic microbead system as described above. Each nude mouse received an adoptive transfer of one-donor equivalent of the $CD4^+$ T-spleen-cell population intravenously (10×10^6 - 15×10^6 cells/recipient). Nude mice were grafted with C57BL/6 corneal allografts within 24 hrs of the adoptive transfer of $CD4^+$ T cells.

Induction of Apoptosis in Cytokine-treated Corneal Endothelial and Epithelial Cells

C57BL/6 corneal endothelial and epithelial cells were washed twice with PBS and plated at 1 $x10^6$ cells per well in a six well plate. Apoptosis was induced by nutrient deprivation through incubation of corneal cell cultures with 1 ml of plain MEM (151). rmIL-17A was added (Sigma-Aldrich) to cell cultures at 10, 100, and 500 ng, with or without 5ng of TGF-β1 (R&D Systems). Cells were collected at 24, 48, and 72 hrs time points and RNA isolated.

Flow Cytometry

The antibodies used for flow cytometric analyses included FITC or PerCPCy5.5-conjugated rat anti-mouse CD4 (eBioscience), PE-conjugated anti-mouse Foxp3 (eBioscience), PE-conjugated anti-mouse RorγT (eBioscience), Apc-conjugated rat anti-mouse CD25 (BD Biosciences), Apc-conjugated rat anti-mouse CTLA-4 (eBioscience), Apc-onjugated rat anti-mouse GITR (eBioscience) and Apc-conjugated chicken anti-human TGFβ1 (R&D Systems). For *in vitro* blocking assays, monoclonal anti mouse-CTLA-4, anti mouse-GITR, anti-TGFβ1, 2, 3, rat IgG_{2A} isotype control, and mouse IgG_1 were purchased from R&D systems. All flow cytometric analyses were performed on a FACS Calibur with CellQuest software (BD Biosciences).

CFSE Suppression Assay

Suppression assays were set up as described previously**.** Putative $CD4^+CD25^+$ Tregs were collected from spleens of cornea-grafted mice 3 weeks post transplantation using Treg isolation kits (Miltenyi Biotec). $5x10^4$ $CD4^+CD25^+$ Tregs isolated from corneal allograft acceptors or rejectors were incubated with $1x10^5$ $CD4^+$ CFSE labeled T cells (T_{eff}) from naïve mice. $CD4^+$ T cells from naïve mice were isolated using the mouse CD4 isolation kit (Miltenyi Biotech, Auburn, CA). The cells were boosted *in vitro* with 1 μg/ml of anti-CD3ε Ab (BD Biosciences) for 72 hrs. Following incubation, cells were stained with Apc-conjugated rat anti–mouse CD25 and PerCPCy5.5-conjugated rat anti–mouse CD4. Subsequently, expression of CD25 was assessed on $CFSE^+$ $CD4^+$ T cells. Percent suppression was calculated using the following formula: % suppression = [(% of $CD25^+CFSE^+$ Teff without Tregs - % $CD25^+CFSE^+$ Teff with Tregs)/(% $CD25^+CFSE^+$ Teff without Tregs)] X 100. Activation of anti-CD3ε-stimulated Teff cells without addition of Tregs was considered as a control with 0% suppression. For blocking

experiments, antibodies were used at 100 μg/ml for anti-IL-17A and rat IgG, 10 μg/ml for anti-CTLA-4 and anti-GITRL, and 50 μg/ml for anti-TGFβ1, 2, 3 (R&D Systems). For experiments where rIL-17A was added, co-cultures were supplemented with either 0.5μg/ml or 1μg/ml of the cytokine (Sigma-Aldrich). Transwell experiments were performed in 96-well plates using Transwell cell culture inserts (0.4 μm; Corning, Inc.).

Acceptor CD4CD25 Cytokine ELISA

Spleen cells were harvested from corneal allograft acceptor BALB/c at 3 weeks post transplantation. Splenocytes were fractionated into $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells using Treg isolation kits (Miltenyi Biotec). Purified cell fractions were incubated at $1x10^6$ cells per well with 1 μg/ml anti-CD3ε Ab for 72 hrs at 37ºC in 2 mLs of complete RPMI. ELISAs for IL-17A, IFN-γ, and TNF-α were performed on culture supernatants according to manufacturer's instructions (R&D Systems).

Quantitative Real-Time PCR (qPCR)

Total RNA was isolated from corneal endothelial cells, corneal epithelial cells, and FACS-sorted $CFSE^-$ $CD4^+CD25^+$ Tregs, using the Maxwell 16 Cell LEV Total RNA Purification kit (Promega) following 72 hrs incubation with CFSE-labeled naïve $CD4^+$ T effector cells. Real-time PCR was performed using the RT² First Strand and RT² SYBR Green kits with preformulated primers for FoxP3 (PPM05497F), CTLA-4 (PPM03217E), GITR (PPM03742E) , TGF-β1(PPM02991A), IL-10 (PPM03017B), BAX (PPM02917E), BCL-2 (PPM02918F), BCL-xL (PPM02920E), and GAPDH (PPM05497F) (SA biosciences). The results were analyzed by the comparative threshold cycle method and normalized by GAPDH as an internal control.

Local Adoptive Transfer (LAT) assay

The LAT assay was used to test the efferent suppression and antigen specificity of $CD4^+CD25^+$ Tregs (178). $CD4^+CD25^+$ Tregs were incubated with BALB/c APCs pulsed with C57BL/6 splenocytes and pre-immune effector $CD4^+$ T cells from corneal allograft rejectors in a 1:1:1 ratio. To generate ACAID $CD8^+$ Tregs, BALB/c mice were given an AC injection of non-adherent C57BL/6 spleen cells on day 0 and were immunized subcutaneously (SC) with 1×10^6 C57BL/6 spleen cells 7 days later. On day 14, spleen cells were collected, and $CD8^+$ T cells were enriched by positive selection using rat anti-mouse CD8-conjugated magnetic microbeads (Miltenyi Biotec, Inc.). ACAID $CD8^+$ Tregs were incubated with BALB/c APCs pulsed with C57BL/6 splenocytes and pre-immune effector $CD4^+$ T cells from corneal allograft rejectors in a 1:1:1 ratio. Left and right ear pinnae of naïve BALB/c mice were injected with 20 µl (1×10^6) of the mixed-cell population. The opposite ear was injected with HBSS as a negative control. Ear swelling was measured 24 hrs later to measure DTH.

Anterior Chamber Priming

The technique for transplanting alloantigenic cells into the AC of the mouse eye has been described elsewhere (177). A Hamilton automatic dispensing apparatus was used to dispense 5 µl of cell suspensions into the AC of anesthetized mice. AC inocula consisting of $1X10^5$ C57BL/6 non-adherent splenocytes were injected into the left eyes of BALB/c mice.

Statistical Analysis

The log–rank test was used for statistical analysis of the differences in the tempos of corneal graft rejection from the Kaplan–Meier survival curves (85). Statistical significance for other

experiments was calculated by the two-tailed t-test. Comparisons yielding $p < 0.05$ were considered significantly different.

CHAPTER THREE

RESULTS

Corneal allograft survival in BALB/c mice

To study the mechanisms that lead to allograft tolerance and rejection, our laboratory uses a well-established murine keratoplasty model. The mice used in a typical keratoplasty experiment are mismatched at both major and minor histocompatibility loci. Corneal buttons are excised from C57BL/6 (H-2^b) mice and transplanted onto BALB/c (H-2^d) mice (**Fig. 3A**). Initial clinical scoring of the corneal allografts is performed 7 days post transplantation when the sutures are removed. As described above, the assessment of graft rejection relies on the degree of corneal opacification. A scale of 0 to 3+ (**Fig. 3B**) is used to assess corneal clarity with corneal grafts receiving two successive scores of 3+ considered as rejected. The animals are followed over a period of 60 days within which 50% of the animals will successively reject their corneas to achieve a mean rejection time (MRT) of 35.2 ± 8.0 days and a median survival time (MST) of 52 days. At the 60 day mark, 50% of the mice receiving the corneal allografts will reject their corneas while 50% of the mice will accept the graft which will remain indefinitely clear (**Fig. 3C**).

For the 50% of the mice that reject their corneal allografts, the rejection process is typically thought to involve $CD4^+$ Th1 cells. As described previously, several observations attest to this assertion. Our own data support this hypothesis as $CD4^+$ T cells isolated from corneal allograft rejectors typically produce significant levels of IFN-γ and TNF-α - signature cytokines of the Th1 subset (**Fig. 3D**). Based on these observations, we initially wanted to test the hypothesis that elimination of the Th1 subset abolishes allograft rejection.

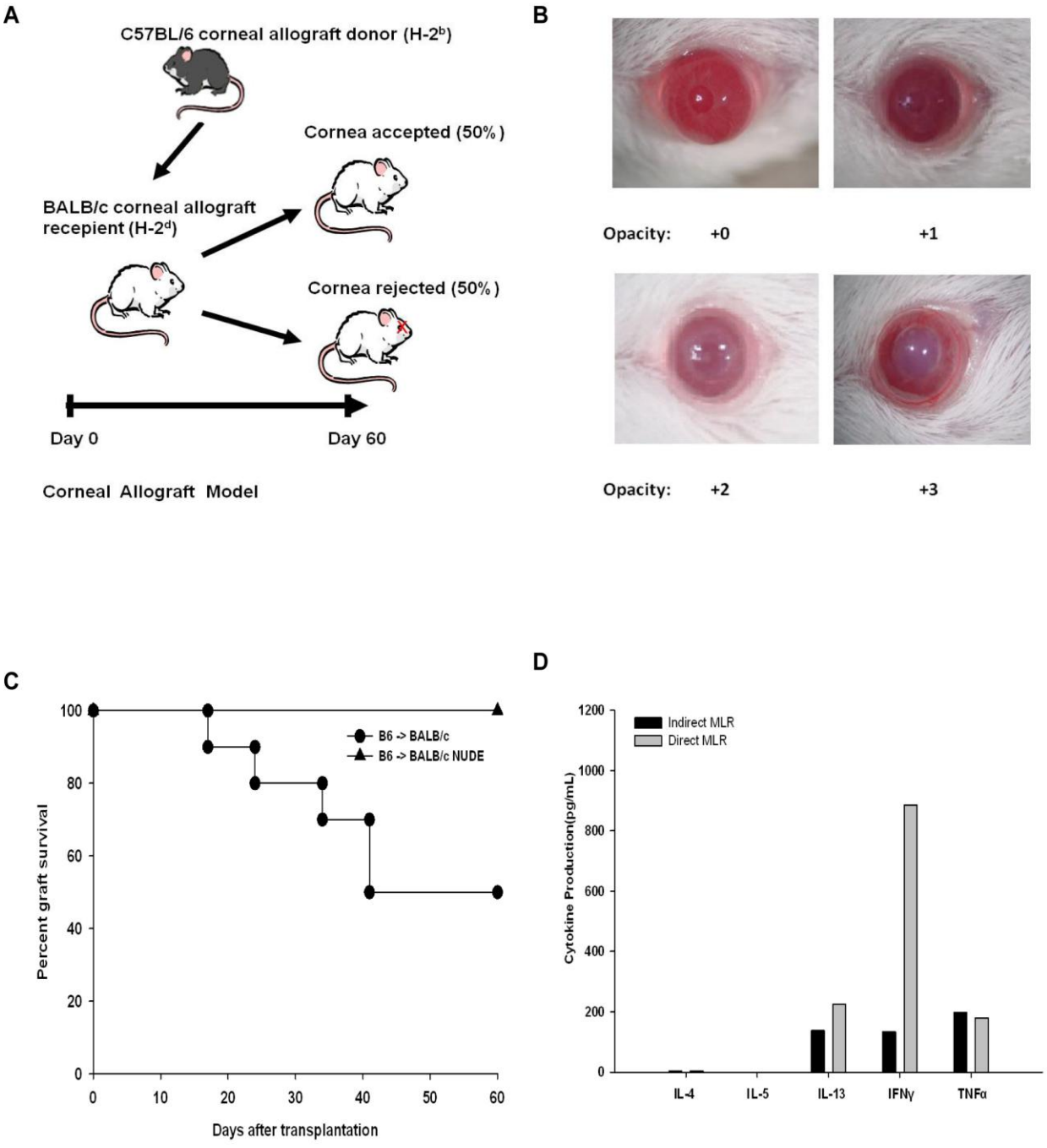

Figure 3. Murine model of keratoplasty. A) Experimental design for murine corneal transplantation with corneas grafted from MHC and miH mismatched C57BL/6 donors to BALB/c recipients. **B)** Clinical assessment of allograft rejection. Corneal graft opacity scores range from 0 to +3, with corneas scoring two successive +3 considered as rejected. **C)** Typical C57BL/6 to BALB/c survival curve with 50% of the grafts enjoying indefinite immune privilege and the other 50% undergoing rejection before day 60. **D)** Cytokine profile of $CD4^+$ T cells from corneal allograft rejectors with a definite Th1 cytokine profile following direct or indirect allostimulation.

ROLE OF IL-17A IN CORNEAL ALLOGRAFT REJECTION

The Th1 subset is not required for allograft rejection

To test the hypothesis that elimination of the Th1 pathway promotes graft survival, we treated BALB/c mice systemically with either anti-IFN-γ mAb or an IgG isotype control Ab given IP twice per week before and after the application of orthotopic C57BL/6 corneal allografts. The isotype control-treated BALB/c hosts rejected 50% of their C57BL/6 corneal allografts with an MRT of 35.2 ± 8.0 days (**Fig. 4**). By contrast, depletion of IFN-γ in BALB/c hosts resulted in a 90% incidence of rejection and an MRT of 22.2 ± 7.3 days (**Fig. 4**). The MST for the anti-IFN-γ-treated group was significantly reduced ($p = 0.014$) compared with that of the IgG isotype control (MST = 23.5 days and 52 days, respectively). This suggests that conventional Th1 cells, as defined by their production of IFN-γ, are not necessary for corneal allograft rejection and implies that either Th2 or Th17 cells are capable of mediating corneal allograft rejection.

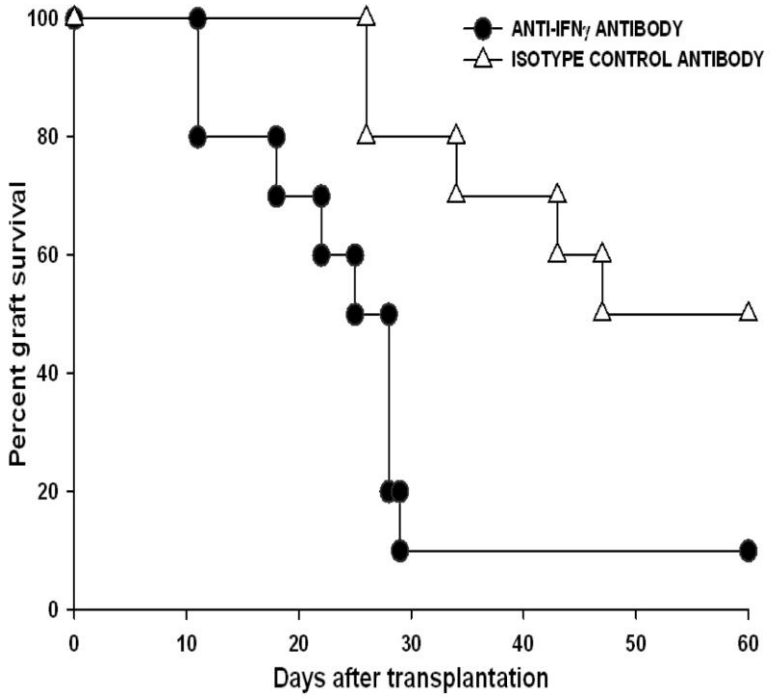

Figure 4. Th1 inhibition exacerbates corneal allograft rejection. C57BL/6 corneal allograft survival in BALB/c mice treated with either anti-IFN-γ or a rat IgG isotype control Ab. BALB/c mice treated with anti-IFN-γ rejected 90% of the C57BL/6 corneal allografts, which had an MST of 23.5 days (N = 10). BALB/c mice treated with a rat IgG isotype control Ab rejected 50% of their grafts (N = 10), with an MST of 52 days. $p < 0.05$ by Kaplan-Meier survival analysis. This experiment was performed three times with similar results giving a total of 30 mice in each group. Syngeneic recipients treated with anti-IFN-γ did not reject their corneal syngrafts (data not shown).

The Th17 subset is not required for allograft rejection

Accordingly, to test the hypothesis that allograft rejection was instead mediated by Th17 cells, BALB/c mice were treated with either monoclonal or polyclonal Abs specific for IL-17A. In multiple experiments, we observed an increased tempo and incidence of corneal allograft rejection in BALB/c mice treated with anti-IL-17A (**Fig. 5**). Mice treated with either monoclonal or polyclonal anti-IL-17A Abs rejected 90% of their corneal allografts, with MRTs of 26 ± 7.9 days and 24.7 ± 12.8 days and MSTs of 24 and 22.5 days, respectively. The rates of corneal allograft rejection between the rat IgG isotype control and anti-IL-17A-treated groups were significantly different ($p < 0.05$).

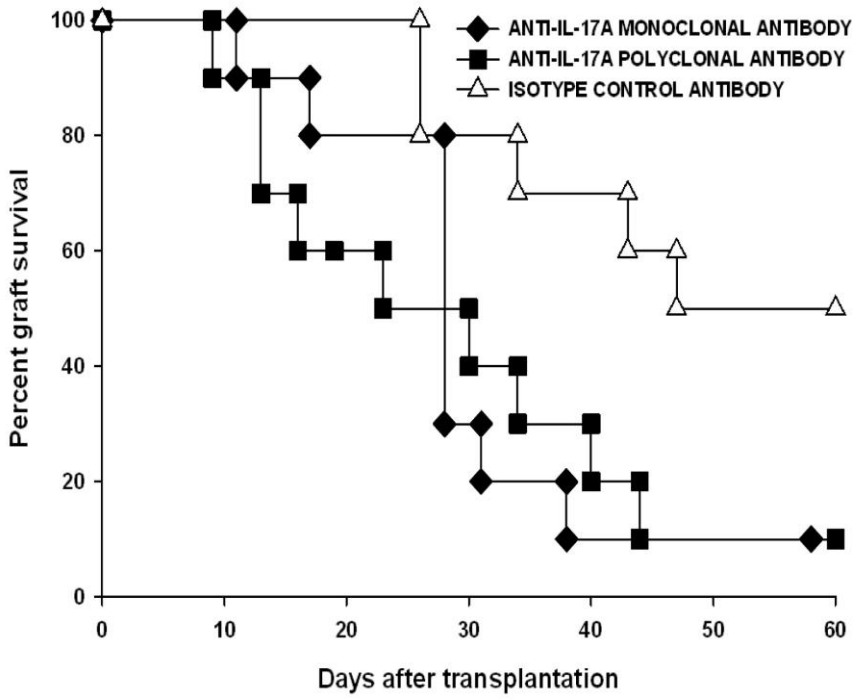

Figure 5. Th17 inhibition exacerbates corneal allograft rejection. C57BL/6 corneal allograft survival in BALB/c mice treated with anti-IL-17A or a rat IgG isotype control Ab. C57BL/6 corneal allografts underwent rejection in 50% of hosts treated with the isotype control IgG (N = 10) and had an MST of 52 days. C57BL/6 corneal allografts transplanted to BALB/c recipients treated with either monoclonal anti-IL-17A or polyclonal anti-IL-17A were rejected in 90% of hosts with an MST of 24 and 22.5 days, respectively (N = 10 in each group). $p < 0.05$ by Kaplan-Meier survival analysis. The experiment was performed three times with similar results giving a total of 30 mice in each group. Syngeneic recipients treated with anti-IL-17A did not reject their corneal syngrafts (data not shown).

The Th1 and Th17 subsets are not required for allograft rejection

Cross-regulation between Th17 and Th1 cell subsets has been previously suggested (53, 72). Therefore, we tested the hypothesis that elimination of both Th1 and Th17 cytokines would enhance graft survival. BALB/c mice were simultaneously treated with anti-IL-17A and anti-IFN-γ monoclonal Abs prior to and after the application of corneal allografts. Treatment with both Abs did not prevent allograft rejection but instead resulted in a 90% incidence of rejection with an MRT of 30 ± 11 days and an MST of 26 days (**Fig. 6**). The tempo of rejection was significantly different compared with that of the rat IgG isotype Ab-treated group ($p = 0.04$). Importantly, neither anti-IFN-γ nor anti-IL-17A Ab treatment affected the survival of syngeneic BALB/c corneal grafts. To our knowledge, these results are the first to demonstrate that elimination of the signature cytokines for the Th1 and Th17 T cell subsets abolishes ocular immune privilege and exacerbates corneal allograft rejection.

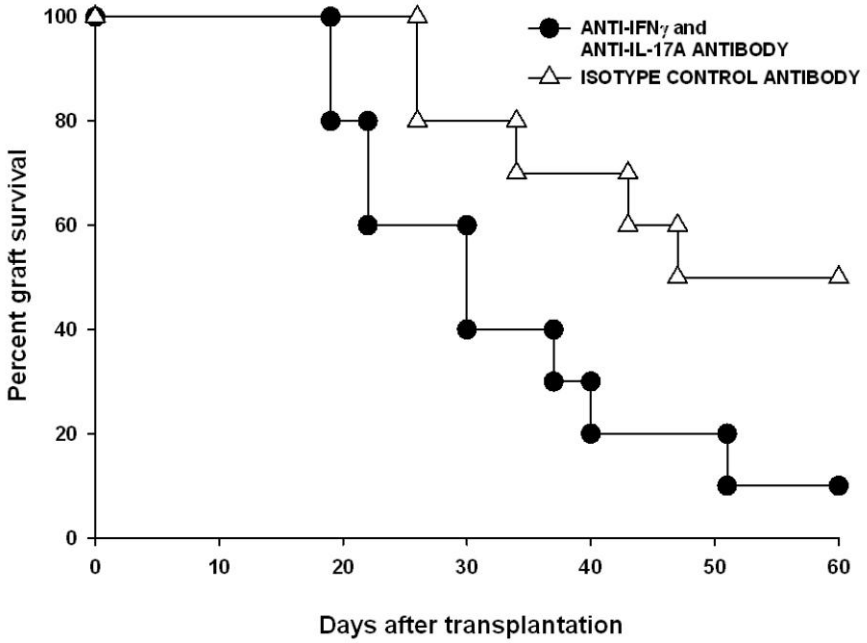

Figure 6. Th17 and Th1 inhibition exacerbates corneal allograft rejection. C57BL/6 corneal allograft survival in BALB/c mice treated with anti-IL-17A and anti-IFN-γ or a rat IgG isotype control Ab. C57BL/6 corneal allografts underwent rejection in 50% of the untreated BALB/c mice (N = 10) and had an MST of 52 days. 90% of C57BL/6 corneal allografts were rejected by the BALB/c mice treated with anti-IL-17A and anti-IFN-γ with an MST of 26 days (N = 10). $p < 0.05$ by Kaplan-Meier survival analysis between cytokine-depleted groups and rat IgG isotype control-treated allograft recipients. These experiments were performed three times with similar results giving a total of 30 mice in each group.

Depletion of the Th17 cytokine does not exaggerate DTH responses

Corneal allograft rejection is closely correlated with the development of DTH responses to donor alloantigens (27, 33-35, 179). Based on the earlier observations that the depletion of IL-17A exacerbated the incidence and tempo of corneal allograft rejection, we performed additional experiments to address the possibility that mice treated with anti-IL-17A might develop exaggerated DTH responses to donor alloantigens. In these experiments, mice were treated with either a rat IgG isotype control or anti-IL-17A on days -4, -2 prior to allograft transplantation, and twice per week during the course of the experiment. BALB/c mice were immunized SC with C57BL/6 splenocytes on day 0 and challenged with mitomycin C-treated C57BL/6 splenocytes in the ear pinnae 14 days later. Mice treated with anti-IL-17A did not display an exaggerated DTH response (**Fig.7A**). To confirm that anti-IL-17A treatment did not promote an exaggerated DTH response in corneal allograft recipients, rat IgG isotype control and anti-IL-17A-treated BALB/c corneal allograft rejector mice were challenged with mitomycin C-treated C57BL/6 splenocytes in the ear pinnae 14 days post-allograft rejection. As in SC immunized mice, corneal allograft rejector mice treated with anti-IL-17A did not develop DTH responses that were any greater than those of similar mice treated with the isotype control IgG (**Fig. 7B**).

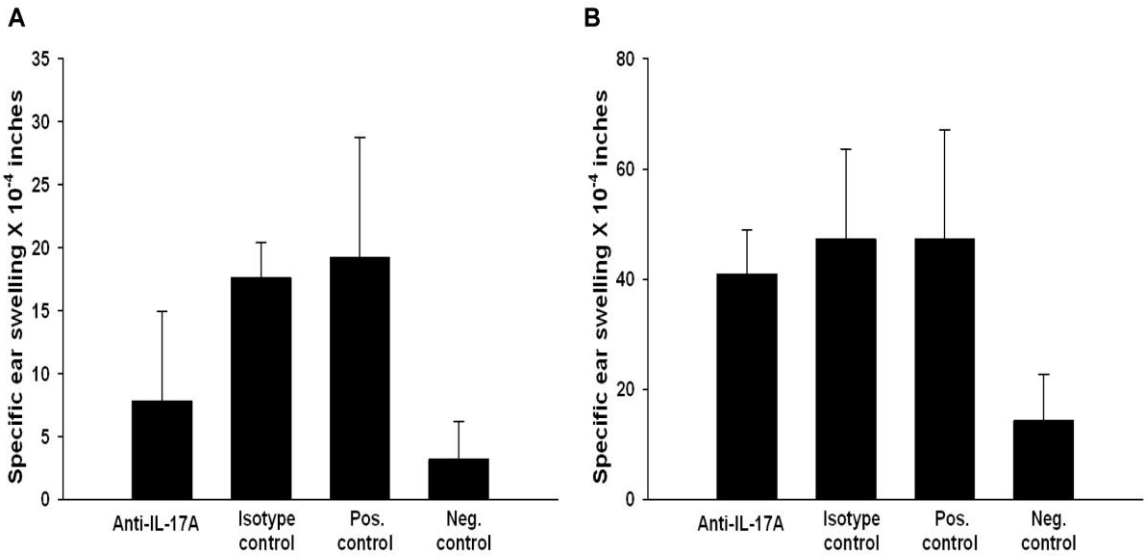

Figure 7. Anti-IL-17A treatment does not exacerbate DTH. A) BALB/c animals were treated with either anti-IL-17A or rat IgG isotype control on days -4, -2 prior to, and twice per week over 2 weeks after SC immunization with C57BL/6 splenocytes on day 0. On day 14, mice were ear challenged with mitomycin C-treated C57BL/6 splenocytes. Negative control animals received an ear challenge only, and positive control animals were immunized SC and received an ear challenge. Each group consisted of five animals. This experiment was performed twice with similar results. Results in all three groups differed significantly from those in the negative control group ($p < 0.05$). Results in the anti-IL-17A Ab-treated group were not significantly different from those of the positive control ($p < 0.05$). **B)** Anti-IL-17A or rat IgG isotype control-treated BALB/c mice with rejected C57BL/6 corneas received an ear challenge with mitomycin C-treated C57BL/6 splenocytes 14 days post rejection. Negative control animals only received an ear challenge, and untreated rejector mice that received an ear challenge 14 days post-rejection served as positive controls. Each group represents five animals, and the experiment was performed once. All results are expressed as mean ear swelling ± SD. Results in all three groups differed significantly from those in the negative control group ($p < 0.05$). Results in the anti-IL-17A Ab-treated group were not significantly different from those in the positive control ($p < 0.05$).

Depletion of IL-17A and IFN-γ promotes emergence of Th2 alloimmune responses

In non-manipulated hosts, corneal allograft rejection was characterized by the production of the Th1 cytokine IFN-γ (**Fig. 8A**). $CD4^+$ T cell production of IL-4 and IL-5 was barely detectable, whereas moderate levels of IL-13 and small quantities of IL-17A and TNF-α were found. To confirm that disabling the Th1 subset with anti-IFN-γ did not exacerbate rejection via the Th17 lineage, we evaluated the cytokine profile of $CD4^+$ T cells that were collected from anti-IFN-γ-treated graft rejector mice and stimulated *in vitro* with C57BL/6 alloantigens in an MLR. The cytokine profile of the allospecific $CD4^+$ T cells indicated a preferential production of the Th2 cytokines IL-4, IL-5, and IL-13, and negligible expression of IL-17A, IFN-γ, and TNF-α (**Fig. 8B**). Mice treated with anti-IL-17A showed a similar cytokine profile, implicating Th2 cells in corneal allograft rejection in hosts lacking IL-17A (**Fig. 8C**). Although IL-17A is known to cross-regulate IFN-γ, *in vivo* treatment with anti-IL-17A Abs did not result in an increased production of IFN-γ (**Fig. 8C**). Thus, disabling the Th1 and Th17 alloimmune responses favors the expression of Th2-mediated corneal allograft rejection. Collectively, these results support the notion that IL-17A and IFN-γ can independently cross-regulate the activity and cytokine secretion of Th2 cells. Based on the Th2 cytokine profiles observed from the MLRs, we hypothesized that the rejected corneas of anti-IFN-γ-treated and/or anti-IL-17A-treated BALB/c corneal allograft rejector mice would display a predominant infiltration of Th2 cells and eosinophils into the graft rejection site. To assess infiltration of Th2 cells within the rejection site, we used Abs specific for T1/ ST2, which is expressed on Th2 cells but not Th1 cells (44, 180). Mononuclear cells infiltrating the corneas of anti-IL-17A-treated animals stained positively for the T1/ST2 antigen (**Fig. 9A-D**), yet no significant eosinophilic infiltrate was detected at the rejection site.

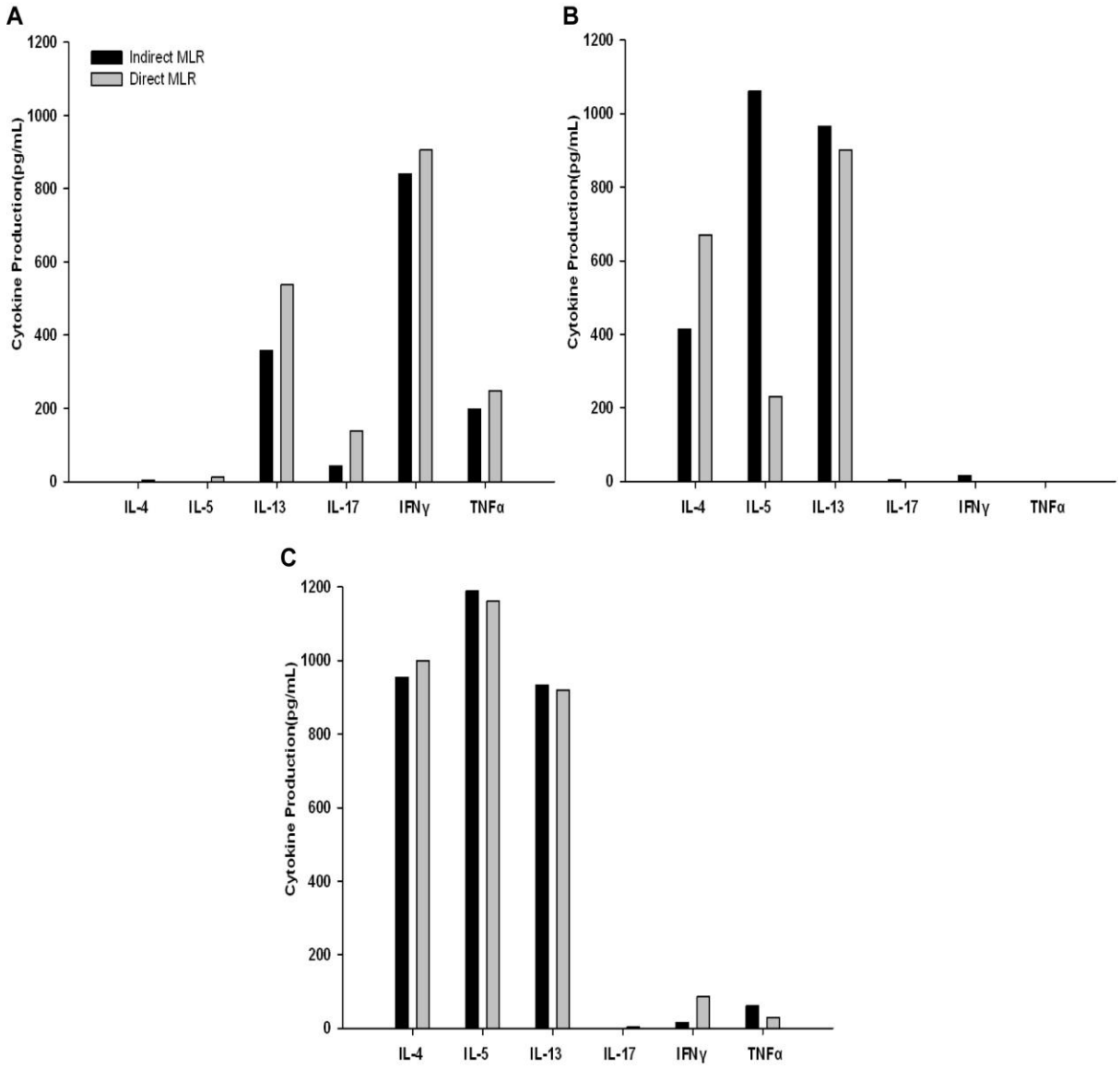

Figure 8. Th1, Th2, and Th17 cytokine production by CD4$^+$ spleen cells from corneal allograft rejector mice. CD4$^+$ spleen cells were isolated from BALB/c mice 4-7 days after their rejection of C57BL/6 corneal allografts. CD4$^+$ spleen cells were stimulated with C57BL/6 alloantigens for 5 days in either direct or indirect MLR cultures. **A)** CD4$^+$ cell supernatants from BALB/c mice treated with rat IgG isotype control Ab. **B)** CD4$^+$ cell supernatants from BALB/c mice treated with anti-IFN-γ Ab. **C)** CD4$^+$ cell supernatants from BALB/c mice treated with anti-IL-17A Ab. Six mice were in each group. This experiment was performed 6 times with similar results.

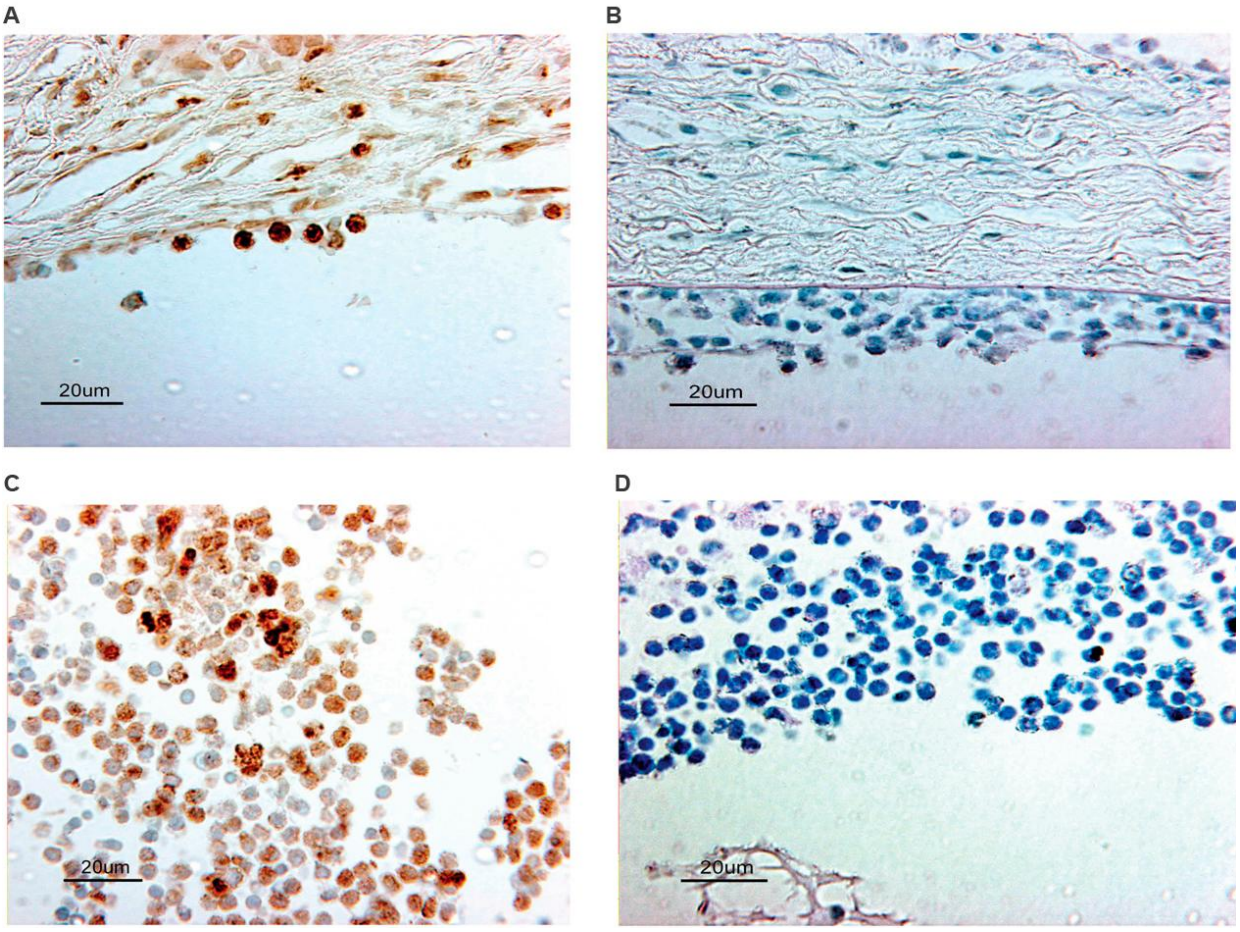

Figure 9. Histological analysis of rejected allografts from anti-IL-17A-treated animals. A) Th2 (T1/ST2^{+}) cells in the corneal stroma and adhering to the corneal endothelium during allograft rejection in anti-IL-17A–treated hosts. **B)** Isotype control Ab tested on same tissue sample shown in **A** reveals no Ab staining. **C)** Th2 (T1/ST2^{+}) cells in the anterior chamber of rejected corneal allograft in an anti-IL-17A-treated BALB/c mouse. **D)** Isotype control Ab tested on same tissue sample shown in C. All specimens were counterstained with methyl green.

The Th2 pathway is sufficient to mediate corneal allograft rejection

The results up to this point suggested that the exacerbation of corneal allograft rejection associated with depletion of either IL-17A or IFN-γ was the result of a Th2 alloimmune response. To explore whether Th2 cells could independently cause graft rejection, we collected $CD4^+$ T cells from anti-IL-17A-treated mice that had rejected their corneal allografts (MRT = 16.7 ± 4.7 days; MST = 13 days) and transferred them into naive BALB/c nude mice. Nude mice that received adoptively transferred $CD4^+$ T cells rejected 100% of their C57BL/6 corneal allografts with an MRT of 23.6 ± 9.2 days and an MST of 17 days (**Fig. 10A**). Immunohistochemical analysis of the rejected corneas revealed a significant infiltration of $T1/ST2^+$ mononuclear cells (**Fig. 10B, 10C**). The transferred $CD4^+$ T cells were predominantly of the Th2 phenotype as confirmed by their preferential production of the Th2 cytokines when confronted with C57BL/6 alloantigens in both the indirect and direct MLRs (**Fig. 11A**). $CD4^+$ T cells collected from the adoptively transferred nude mice following allograft rejection were further phenotyped by cytokine analysis of MLR supernatants (**Fig. 11B**). Similar to the adoptively transferred $CD4^+$ T cell population, the $CD4^+$ T cells in the nude rejector mice predominantly produced Th2 cytokines. Interestingly, significant levels of IFN-γ and TNF-α were also produced by the $CD4^+$ cells from nude mice, suggesting the emergence of a Th1 response. Several explanations could account for this observation. For instance, the adoptively transferred $CD4^+$ T cell might have converted from the Th2 to the Th1 lineage or might have originally contained a Th1 population that was suppressed in the donors. Additionally, the IFN-γ and TNF-α might originate from $CD4^+$ expressing non-T cells present in the hosts. Either way, the contribution of this Th1 cell subset in allograft rejection in the Th1 hosts will need further evaluation.

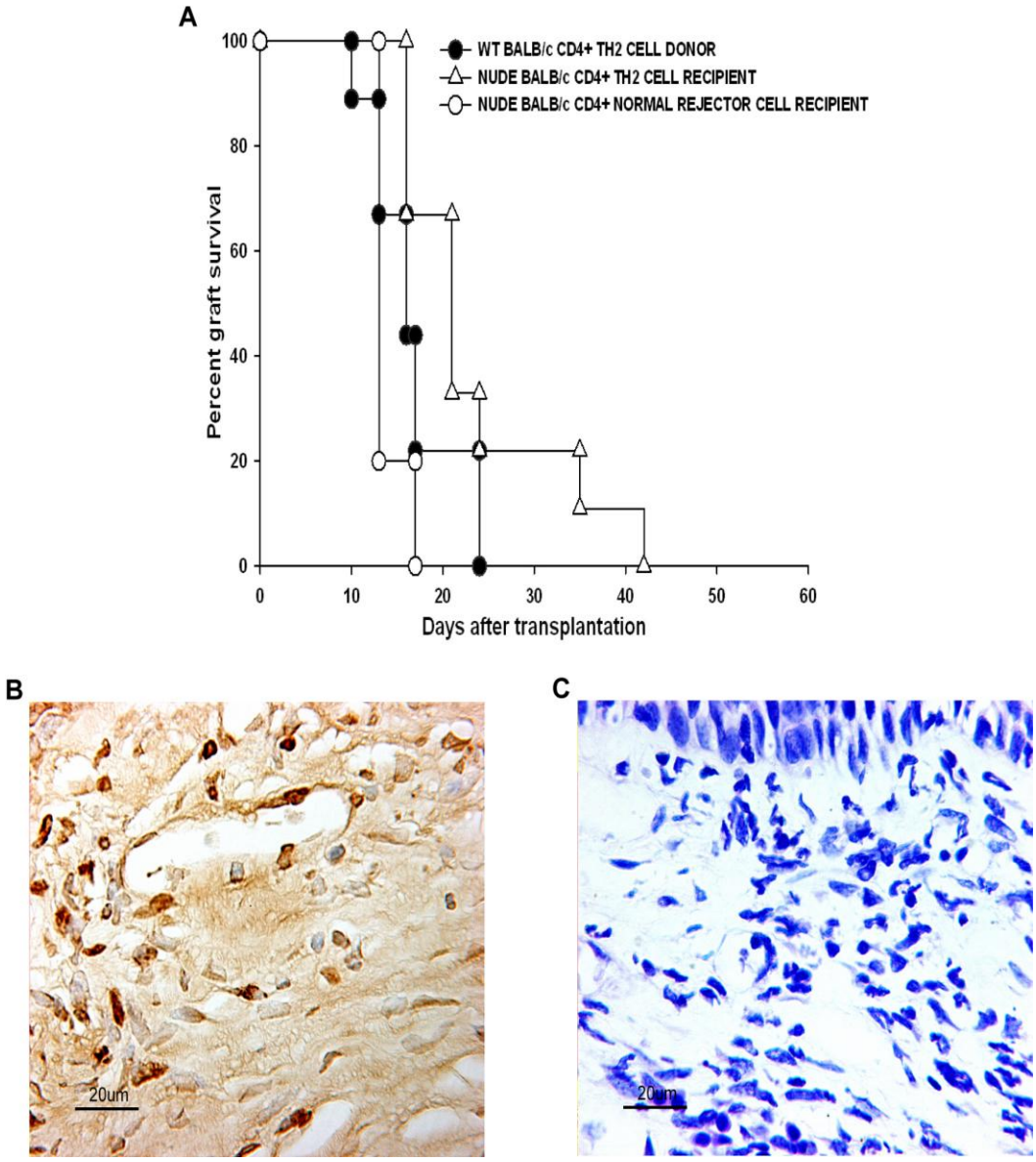

Figure 10. The Th2 lineage can independently mediate corneal allograft rejection. Corneal allograft survival in anti-IL-17A-treated nude BALB/c mice after adoptive transfer of $CD4^+$ T splenocytes from corneal allograft rejector BALB/c mice treated with anti-IL-17A. **A)** Corneal allograft rejection in anti-IL-17A-treated BALB/c mice and in BALB/c nude mice that received adoptively transferred $CD4^+$ T cells from either anti-IL-17A-treated or untreated BALB/c corneal allograft rejector donors. C57BL/6 corneal allografts underwent rejection in 100% of the nude BALB/c recipient hosts that received $CD4^+$ T cells from either anti-IL-17A donors (N = 9) or untreated donors (N = 5) and had MSTs of 17 and 7 days, respectively. WT BALB/c $CD4^+$ T cell donors rejected their corneas with an MST of 13 days (N = 9). $p > 0.05$ by Kaplan-Meier survival analysis. **B)** T1/ST2 expression in cell infiltrates of rejected corneal allografts from nude mice that received adoptively transferred $CD4^+$ T cells. **C)** Isotype control staining of the same corneas. All specimens were counterstained with methyl green.

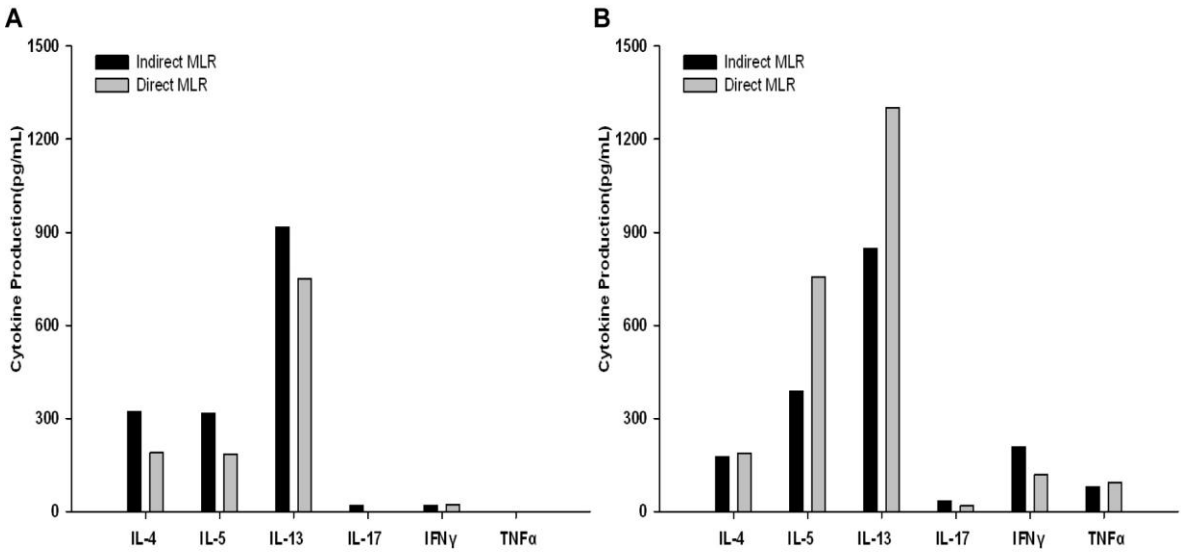

Figure 11. Cytokine profile of Th2 cells prior to and following adoptive transfer into nude mice. A) Cell supernatant cytokine profiles of $CD4^+$ cells that were isolated from anti-IL-17A-treated corneal allograft rejector donor mice and adoptively transferred to nude BALB/c mice. This experiment was performed 4 times with similar results. **B)** Cell supernatant cytokine profiles of splenic $CD4^+$ cells that were isolated from anti-IL-17A-treated corneal allograft rejector BALB/c nude recipients. This experiment was performed 4 times with similar results.

Elimination of the Th2 lineage decreases the tempo of allograft rejection in anti-IL-17A-depleted hosts

The previous results suggest that anti-IL-17A treatment elicited an immune deviation that favored the emergence of allospecific Th2 cells that were sufficient to mediate corneal allograft rejection. With this in mind, we examined the fate of corneal allografts transplanted into STAT6 KO mice, which do not generate IL-4-mediated functions including Th2 cell differentiation (181, 182). Accordingly, STAT6 KO mice on a BALB/c background were grafted with C57BL/6 corneal allografts. Similar to WT mice, the isotype-treated STAT6 KO mice rejected 40% of their corneal allografts (**Fig. 12A**). However, when the STAT6KO mice were treated with a combination of anti-IFNγ and anti-IL-17A, instead of experiencing the 10% graft survival observed with WT recipients (MST = 26 days), the percentage of surviving grafts in the cytokine-depleted STAT6KO mice increased to 87.5% with an MST of 60 days ($p < 0.05$).These results support the hypothesis that blockade of the Th17 and Th1 pathways favors the emergence of a Th2-mediated rejection of corneal allografts.

To further investigate the role of IL-17A in mediating corneal immune privilege in the STAT6 KO hosts, we treated the STAT6 KO BALB/c mice systemically with either anti-IL-17A mAb or an IgG isotype control Ab and followed the mice over a period of 120 days. Over the first 60 days following allograft transplantation, isotype control-treated animals rejected 40% of the corneal allografts and remained at 60% survival over the next 60 days, typical of wild-type recipients. Interestingly, hosts treated with anti-IL-17A rejected 80% of their corneal allografts at the end of the 120 days (**Fig. 12B**). Although not statistically significant, this increased incidence of rejection suggested that IL-17A served additional roles in maintaining immune privilege in addition to cross-regulating the Th2 response.

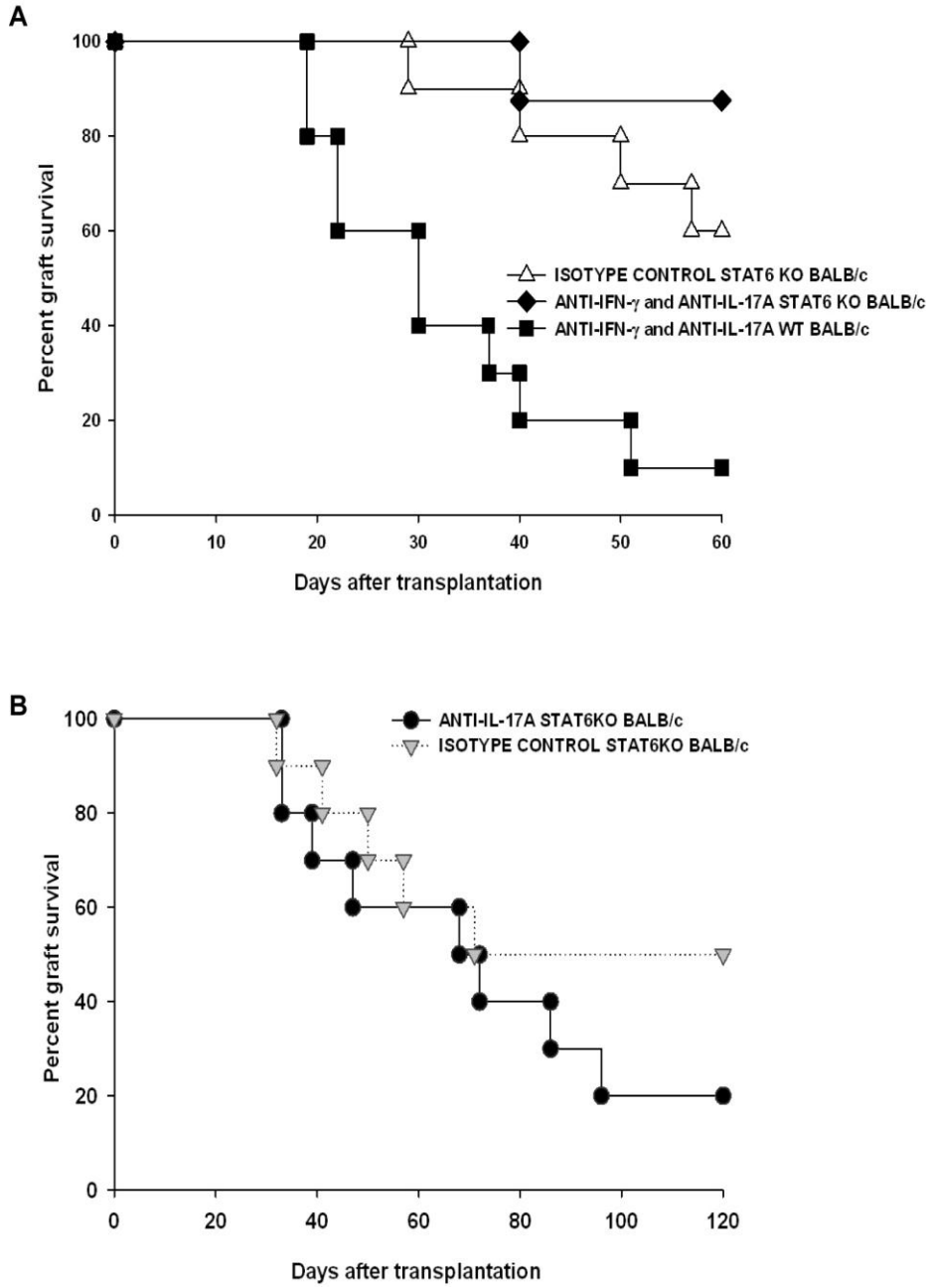

Figure 12. IL-17A-deficient STAT6 KO mice retain corneal immune privilege. A) Effect of anti-IL-17A and anti-IFN-γ Ab treatment on corneal allograft survival in Th2-impaired hosts. STAT6 KO BALB/c mice were treated with anti-IL-17A and anti-IFN-γ mAbs and transplanted with C57BL/6 corneal allografts. For comparison, WT BALB/c mice were similarly treated with anti-IL-17A and anti-IFN-γ mAbs and transplanted with C57BL/6 corneal allografts. WT mice treated with anti-IL-17A and anti-IFN-γ (N = 10) Abs rejected 90% of their corneal allografts with an MST of 26 days. By contrast, only one of eight STAT6 KO mice treated with anti-IFN-γ and anti-IL-17A Abs rejected its corneal allograft. $p < 0.05$ by Kaplan-Meier survival analysis between cytokine-depleted WT and STAT6KO groups .**B)** STAT6 KO BALB/c mice treated with either anti-IL-17A or the IgG isotype control were followed for 120 days. STAT6 KO BALB/c mice treated with rat IgG rejected 50% of their corneal allografts with an MST of 94 days compared to the anti-IL-17A-treated STAT6KO mice which rejected 80% of their corneal allografts with an MST of 67 days.($p > 0.05$)

ROLE OF IL-17A IN CORNEAL IMMUNE PRIVILEGE

Our original hypothesis for this study proposed that the Th17 subset and its effector cytokine IL-17A were necessary for corneal allograft rejection. However, inhibition of the Th17 subset by systemic depletion of its cytokine abolished corneal immune privilege and exacerbated the incidence and tempo of allograft rejection. In view of the current findings, the second specific aim hypothesized that IL-17A was required for corneal immune privilege.

Our initial investigations explored the potential local roles for IL-17A at the corneal allograft-host interface. Several reports had previously described the beneficial effects of another inflammatory molecule, IFN-γ, on enhancing corneal immune privilege (93, 157). Thus, the first set of experiments looked into the changes in the expression pattern of endogenous corneal surface molecules that assist in stifling the efferent phase of the immune response following treatment with IL-17A. For the second sub-aim, IL-17A-mediated modulation of the expression of either pro- or anti-apoptotic molecules was tested in studies that examined a possible association of this cytokine with the evasion of apoptosis (183).

In addition to local immunoregulation, systemic immunosuppression mediated by $CD4^+CD25^+$ Tregs is required for sustaining corneal immune privilege. Based on several reports in the literature that indentified IL-17A-producing $CD4^+CD25^+$ Tregs (172, 173), the latter part of this section examined the possible interplay between $CD4^+CD25^+$ Tregs and IL-17A in maintaining corneal allograft survival.

IL-17A does not upregulate expression of suppressive molecules produced by corneal cells

Several groups, including ours, have previously reported a beneficial effect for the inflammatory cytokine, IFN-γ in the context of corneal immune privilege (93, 157, 171). Indeed, Hori *et al.* had previously demonstrated that PD-L1 expression by corneal endothelial cells was necessary for corneal immune privilege (158). IFN-γ-deficient mice had significantly impaired expression of the molecule and could not support corneal immune privilege. Similarly, Larkin *et al.* also showed that IFN-γ could significantly enhance expression of IDO in corneal endothelial cells, thereby inhibiting the efferent phase of the immune response (93). Lastly, our group also described a decreased expression of TRAIL on the corneal tissues in IFN-γ-deficient mice (156).

Based on the observation that both IL-17A and IFN-γ are inflammatory cytokines, and depletion of either leads to the abolition of corneal immune privilege, along with the additional findings described above, we investigated whether IL-17A could be promoting corneal immune privilege by upregulating the expression of suppressive molecules expressed by corneal cells. Accordingly, expression of PD-L1, TRAIL, FasL, and IDO was evaluated in IL-17A-treated C57BL/6 corneal endothelial and epithelial cells by qPCR analysis (**Fig. 13**). IFN-γ-treated corneal cells were used as a positive control (**Fig. 13**). No significant upregulation in the expression of the molecules was detected. Subsequent analysis looking at changes in protein expression of the membrane molecules by FACS analysis did not show any changes in their expression (**Fig. 14**).

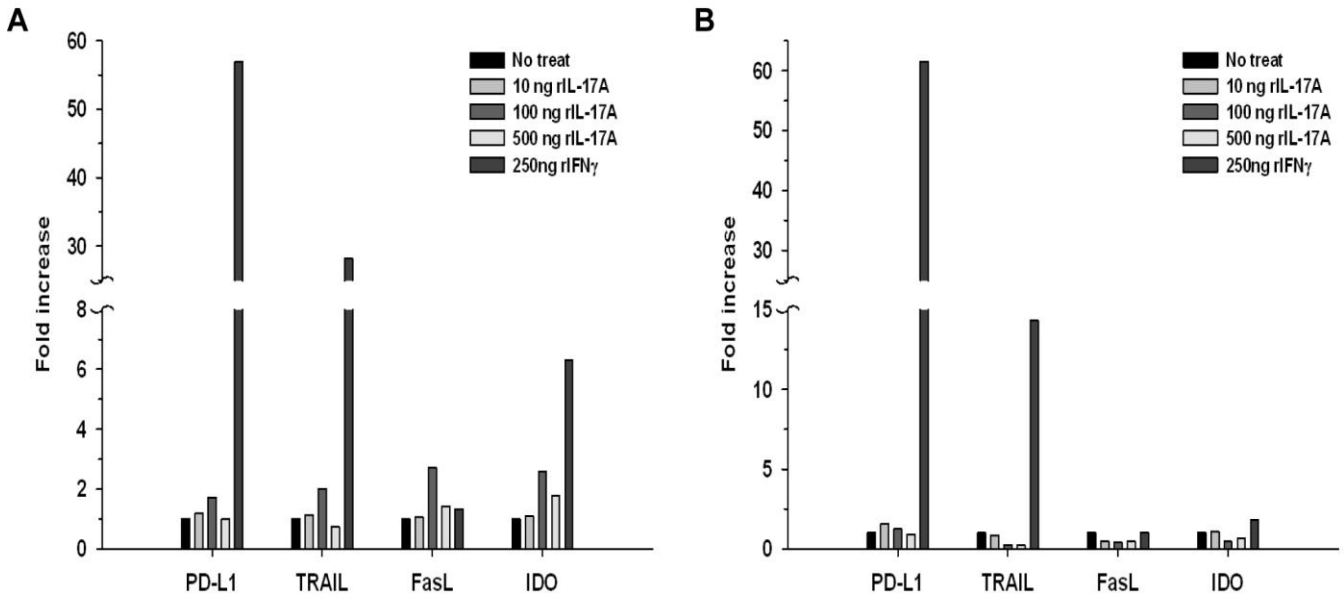

Figure 13. IL-17A does not increase mRNA expression of PD-L1, TRAIL, FasL, or IDO in corneal cells. C57BL/6 corneal **A)** endothelial and **B)** epithelial cells were washed twice with PBS and plated at 1 x10^6 cells per well in a six well plate. rmIL-17A was added to cell cultures at 10, 100 and 500 ng/ml. IFN-γ-treated corneal cells were used as a positive control. At 72 hrs, cells were collected, RNA was extracted, and qPCR for PD-L1, TRAIL, FasL, and IDO was performed. Experiments were performed twice with similar results.

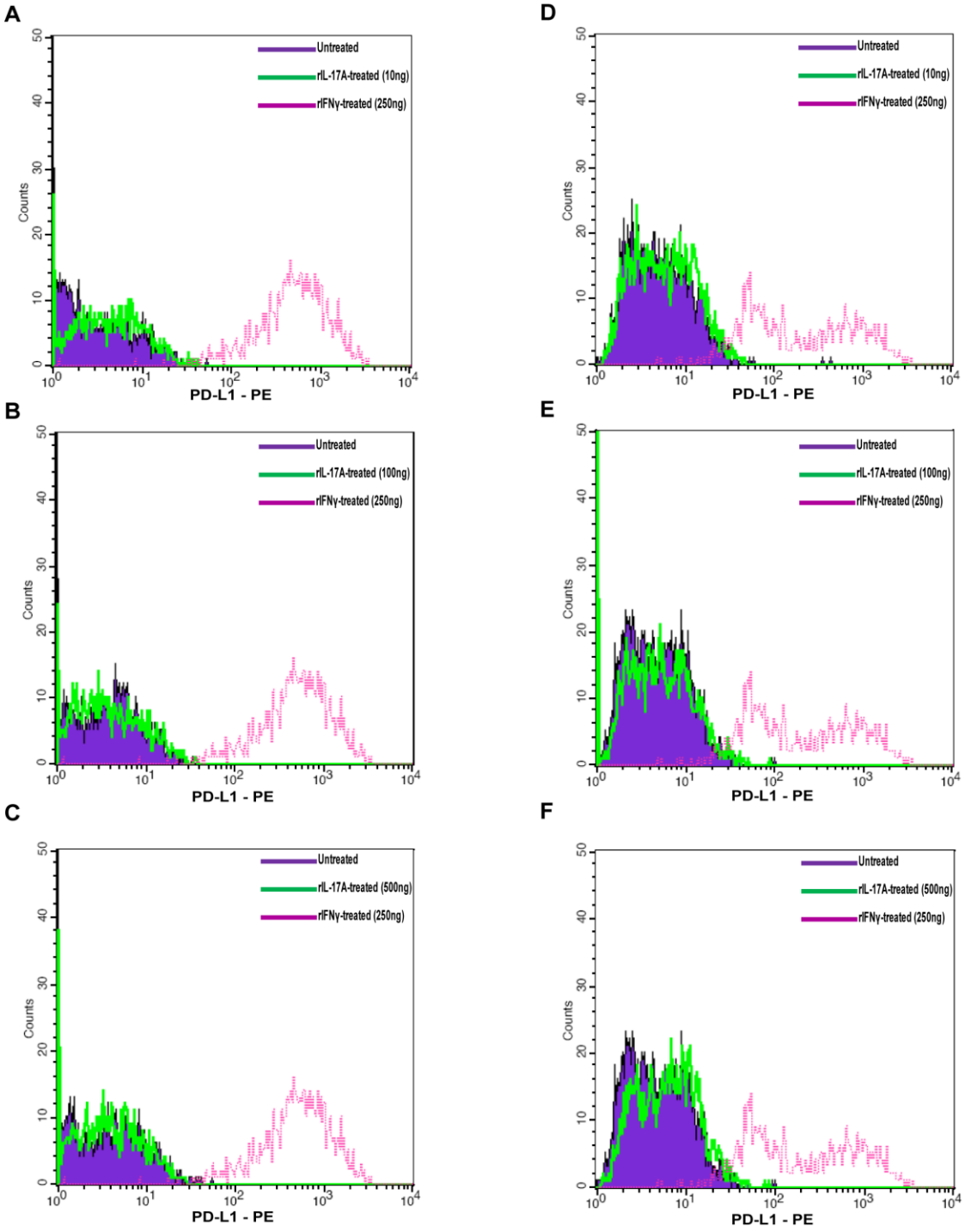

Figure 14. IL-17A does not increase protein expression of PD-L1 on corneal cells. C57BL/6 corneal **A-C)** endothelial and **D-F)** epithelial cells were washed twice with PBS and plated at 1 x10^6 cells per well in a six well plate. rmIL-17A was added to cell cultures at 10, 100 and 500 ng/ml. Corneal cells treated with 250ng of rmIFN-γ were used as a positive control. Cells were collected at 24, 48 and 72 hrs time points at which immunostaining for PD-L1, TRAIL and FasL was performed. Results described here are representative of the 72 hrs time point for PD-L1. Experiments were performed twice with similar results.

IL-17A does not protect corneal cells from apoptosis

The next series of experiments addressed whether IL-17A could protect the corneal allograft from apoptosis. Studies in our laboratory had previously indicated that factors produced by the iris and ciliary body cells and present in the AH, upregulate BCL-2 gene transcription and protect the corneal endothelial cells from apoptosis (151). Moreover, Nam and coworkers had previously described a synergistic effect of TGF-β1 and IL-17A on the evasion of apoptosis by inhibition of caspase-3 cleavage (183). The fact that the AH bathes the cornea with copious amounts of TGF-β provided a strong rationale upon which to test this hypothesis.

Apoptosis was induced in C57BL/6 corneal endothelial and epithelial cells by nutrient deprivation through incubation of corneal cell cultures with 1 ml of serum-free MEM. Cells were incubated with rmIL-17A at 10, 100, and 500 ng/ml, with or without 5ng of TGF-β1/ml. Cells were collected at 24, 48 and 72 hr time points and RNA was isolated. Corneal cells were also cultured in complete RPMI as a positive control. qPCR analysis was performed and changes in expression of the anti-apoptotic genes, BCL-2 and BCL-xL, and the pro-apoptotic gene, BAX, were examined. The results indicate that IL-17A treatment of corneal endothelial cells did not significantly upregulate either BCL-2 or BCL-xL, but did cause a decrease in the expression of BAX (**Fig. 15A and B**).

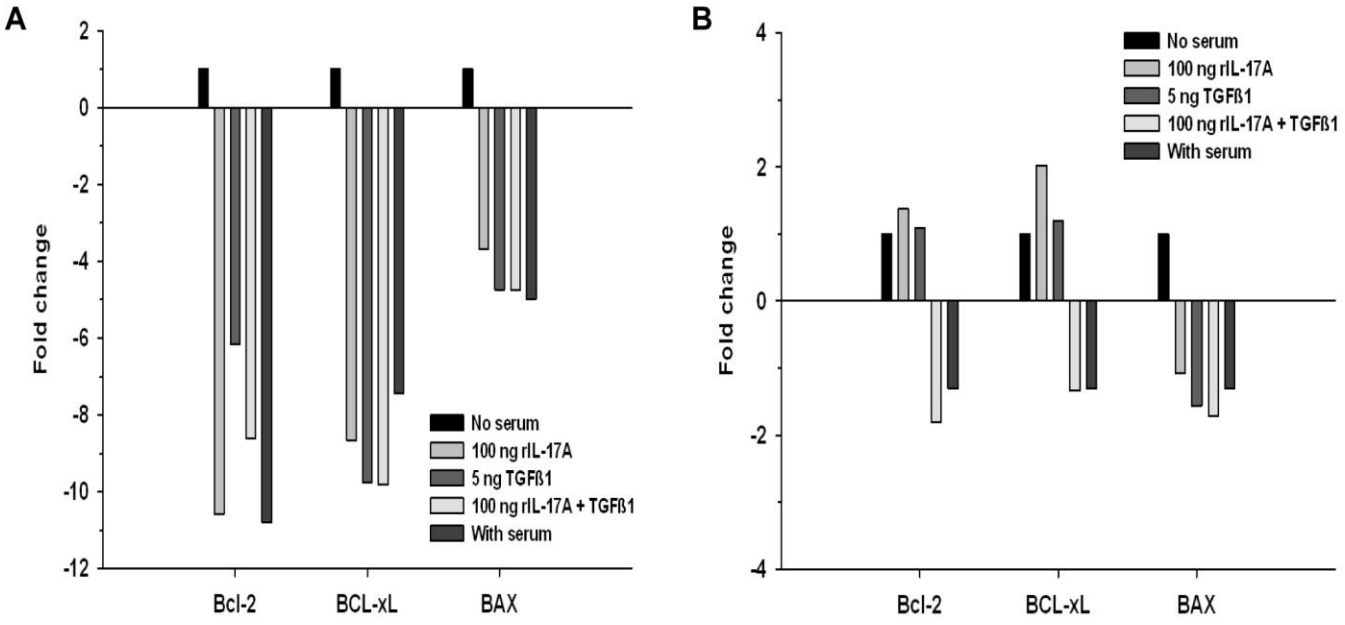

Figure 15. IL-17A-mediated changes in the expression of pro and anti-apoptotic molecules in corneal cells. C57BL/6 corneal **A)** endothelial and **B)** epithelial cells were washed twice with PBS and plated at 1 x10^6 cells per well in a six well plate in serum-free MEM. rmIL-17A was added to cell cultures at 10, 100, and 500 ng/ml, with or without 5ng/ml of TGF-β1 . Corneal cells were also cultured in complete RPMI as a positive control. Cells were collected at 24, 48, and 72 hrs time points at which RNA was isolated. qPCR for BCL-2, BCL-xL, and BAX was performed. Results described here are representative of the 72 hr time point. Experiments were performed twice with similar results.

IL-17A depletion does not cause a decrease in the number of $CD4^+CD25^+$ Tregs

$CD4^+CD25^+Foxp3^+$ Tregs are required for the establishment of corneal immune privilege (85). Based on previous reports and our own observations, we next tested the hypothesis that the increase in graft rejection following depletion of IL-17A was the result of a decrease in the number of $CD4^+CD25^+Foxp3^+$ Tregs (86-88). Splenocytes were collected from graft acceptor, graft rejector, and anti-IL-17A-treated graft rejector mice three weeks post transplantation and flow cytometric analysis was performed to assess the frequency of $CD4^+CD25^+Foxp3^+$ Tregs. The spleen was selected as a source for $CD4^+CD25^+Foxp3^+$ Tregs based on previous experiments in our laboratory which showed that this organ was necessary for corneal immune privilege. No significant difference was observed between the percentage of $CD4^+CD25^+Foxp3^+$ Tregs either as a proportion of the total $CD4^+$ T cells or of the total splenic cells between graft acceptor and rejector mice (**Fig. 16A**). In contrast, the Treg frequency in the anti-IL-17A-treated rejector mice was significantly higher when compared to the graft acceptor mice. The number of $CD4^+CD25^+Foxp3^-$ T cells, commonly viewed as effector cells, from the three differentially treated mice was also assessed. Although no significant difference was observed between the acceptor and rejector mice, the fraction of $CD4^+CD25^+Foxp3^-$ T cells in the anti-IL-17A-treated mice was significantly higher (**Fig. 16B**).

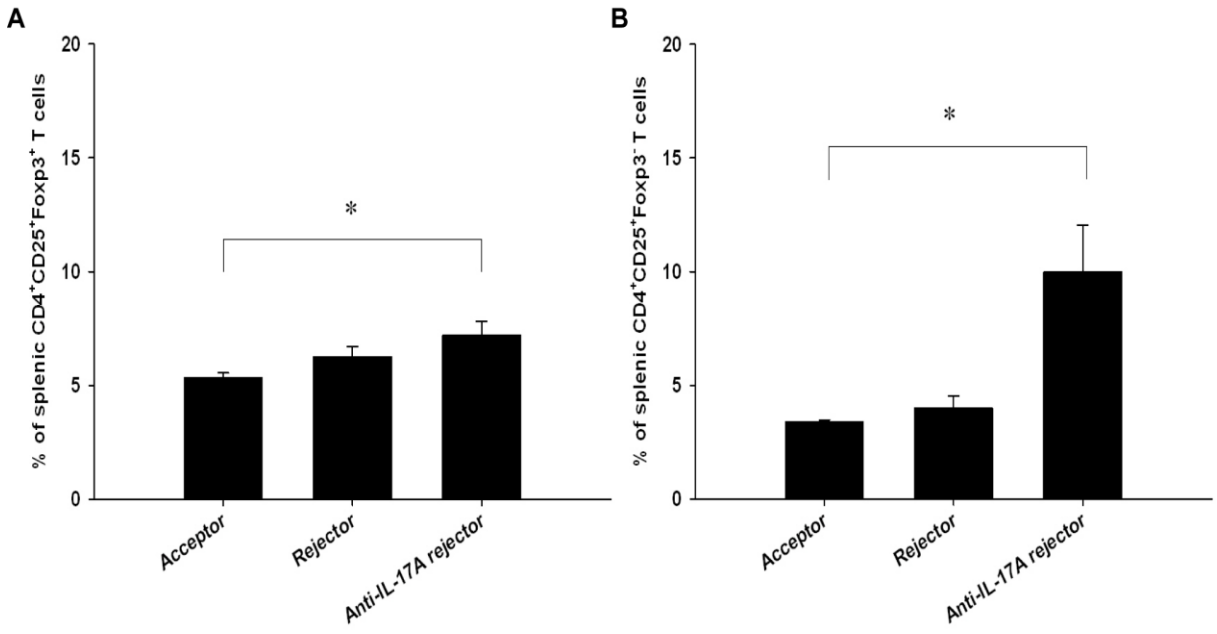

Figure 16. Frequency of $CD4^+CD25^+Foxp3^{+/-}$ Tregs in acceptor, rejector, and anti-IL-17A rejector mice. BALB/c mice were treated with 500 μg of anti-IL-17A antibody or isotype control on days -4 and -2 prior to and twice weekly following corneal transplantation. At day 21, splenocytes were collected from the rat IgG-treated rejectors, survivors, and the anti-IL-17A-treated rejectors. The frequency of **A)** $CD4^+CD25^+Foxp3^+$ Tregs and **B)** $CD4^+CD25^+Foxp3^-$ Teff was subsequently assessed. The experiment was done three times for a total of 9 mice per group.

IL-17A depletion impairs CD4$^+$CD25$^+$ Tregs suppressive abilities

The next series of experiments addressed whether IL-17A is required to induce suppression of CD4$^+$ T effector cells co-cultured with CD4$^+$CD25$^+$ Tregs. CFSE-labeled naïve CD4$^+$ T effector cells were activated using anti-mouse CD3ε antibody and co-cultured with CD4$^+$CD25$^+$ Tregs isolated from either BALB/c corneal allograft rejector or acceptor mice three weeks post transplantation. Suppression of CFSE-labeled CD4$^+$ T effector cell activation by CD4$^+$CD25$^+$ Tregs was determined by quantifying CFSE$^+$ T effector cells expressing CD25. Anti-IL-17A antibody or an IgG isotype control antibody was added to the co-culture assays to determine whether IL-17A was required for CD4$^+$CD25$^+$ Treg-mediated suppression (**Fig. 17A**). CD4$^+$CD25$^+$ T cells collected from corneal allograft rejector mice did not significantly suppress the activation of the naïve CD4$^+$ T effector cells in cultures treated with isotype IgG antibody, whereas, CD4$^+$CD25$^+$ T cells from corneal allograft acceptor mice suppressed the activation of naïve CD4$^+$ T effector cells in the presence of isotype IgG antibody (**Fig. 17B**). Addition of anti-IL-17A antibody to CD4$^+$CD25$^+$ Tregs from corneal allograft acceptor mice and naïve CD4$^+$ T effector cell co-cultures resulted in a significant reduction in the suppression of naïve CD4$^+$ T effector cell activation compared to isotype-treated controls. No difference in suppression was evident between anti-IL-17A-treated and isotype-treated CD4$^+$CD25$^+$ rejector Tregs in CD4$^+$ T effector cell co-cultures.

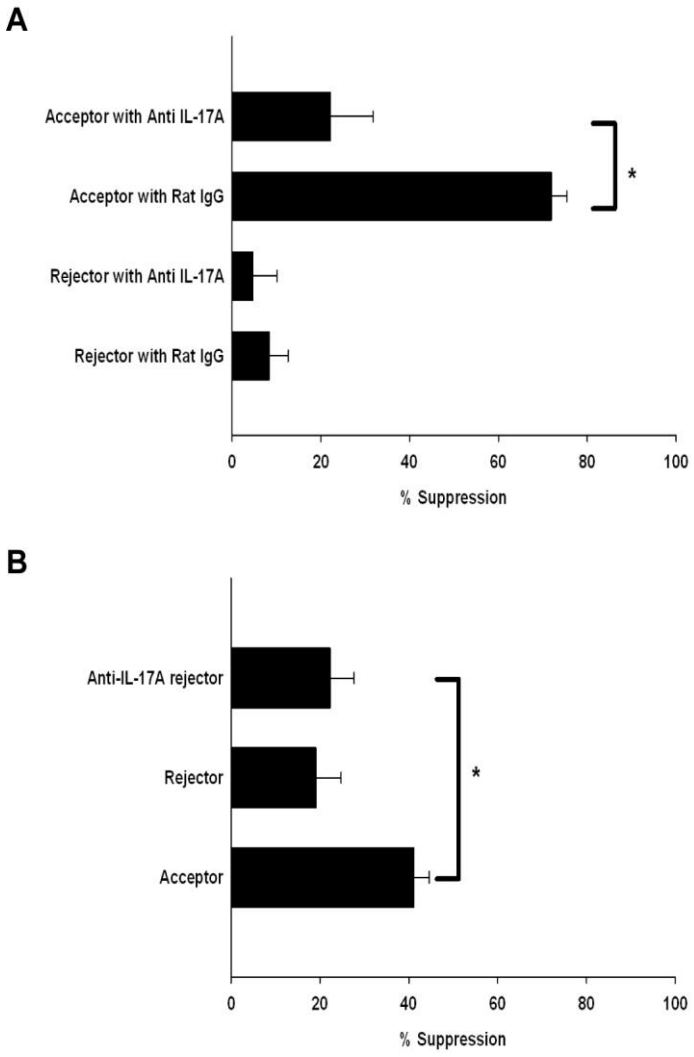

Figure 17. Effect of anti-IL-17A on Treg function. A) *An in vitro* CFSE suppression assay was used to assess the regulatory potential of Tregs from acceptor and rejector mice in the presence of either anti-IL-17A or isotype control IgG. Suppression was based on naïve $CD4^+$ T effector cell expression of CD25 following 72 hrs of co-culture with $CD4^+CD25^+$ Tregs isolated from corneal allograft acceptor mice and in the presence of anti-CD3ε Ab. *$p < 0.05$. N = 3 mice per group (allograft acceptors and allograft rejectors) with results being consistent in two independent experiments. **B)** $CD4^+CD25^+$ Tregs from corneal allograft survivors were collected from isotype control-treated corneal allograft survivors and rejectors, and from anti IL-17A-treated-rejectors 3 weeks post transplantation. Suppression was based on naïve $CD4^+$ T effector cell expression of CD25 following 72 hrs of co-culture with individual $CD4^+CD25^+$ Tregs in the presence of anti-CD3ε Ab (*$p < 0.05$) .

IL-17A is selectively produced by acceptor CD4$^+$CD25$^-$ T cells

Our initial hypothesis proposed that Th17 T cells mediated corneal allograft rejection. However, the weight of evidence shown here indicates the opposite and suggests that IL-17A may contribute to the immune privilege of corneal allografts. To test the hypothesis that IL-17A and IFN-γ production correlates with corneal allograft survival, CD4$^+$ T cells were isolated from corneal allograft acceptor mice and stimulated *in vitro* with C57BL/6 alloantigens. IL-17A production was measured by ELISA in addition to IFN-γ and TNF-α, which had previously been shown to be necessary for the generation and activation of CD4$^+$CD25$^+$ Tregs (184-186). CD4$^+$ T cells from mice that had accepted their corneal allografts produced large amounts of IL-17A and IFN-γ and moderate levels TNF-α (**Fig. 18A**)

Next, we wanted to assess which cell subset within the CD4$^+$ acceptor T cell population was producing IL-17A. The next series of experiments was performed to determine whether CD4$^+$CD25$^+$ Tregs and/or CD4$^+$CD25$^-$ T cells produce IL-17A. Accordingly, CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ T cells from corneal allograft acceptor mice were stimulated with anti-CD3ε antibody or via direct MLR and the culture supernatants were examined by ELISA for the presence IL-17A, IFN-γ, and TNF-α. No detectable levels of IL-17A, IFN-γ, or TNF-α were found in supernatants from stimulated CD4$^+$CD25$^+$ T cells from corneal allograft acceptor mice (**Fig. 18B and C**). By contrast, IL-17A, IFN-γ, and TNF-α were detected in supernatants of CD4$^+$CD25$^-$ T cells. (**Fig. 18B and C**)

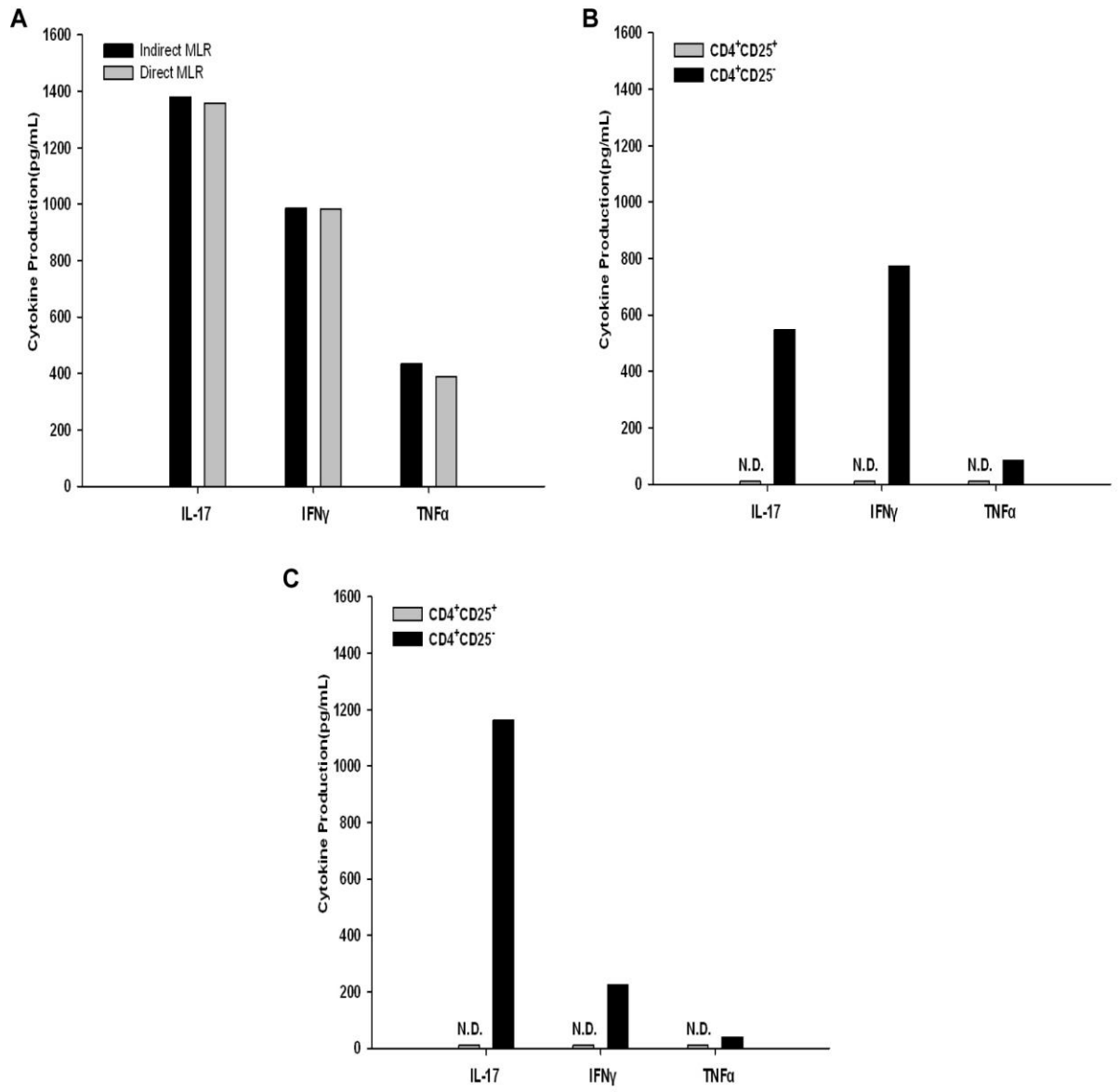

Figure 18. Acceptor $CD4^+$ T cell cytokine profile A) Cytokine production by $CD4^+$ spleen cells from corneal allograft acceptor BALB/c mice. $CD4^+$ spleen cells were isolated from BALB/c mice bearing clear C57BL/6 corneal allografts on day 21 post-transplantation and were stimulated *in vitro* with C57BL/6 alloantigens in either a direct or indirect MLR. Culture supernatants were evaluated for cytokines by ELISA. Six mice were used for the analysis, which was performed two times with similar results. **B)** Cytokine profile of $CD4^+CD25^+$ and $CD4^+CD25^-$ splenic T cells from corneal allograft acceptor BALB/c mice. Acceptor $CD4^+$ T cells were collected as described above and fractionated into $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. Cells were subsequently stimulated via a direct MLR for 72hrs, after which an ELISA was performed on the culture supernatants. 6 corneal allograft acceptor mice were used in 2 separate experiments. **C)** Cytokine profile of $CD4^+CD25^+$ and $CD4^+CD25^-$ splenic T cells from corneal allograft acceptor BALB/c mice. Acceptor $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were stimulated with anti-CD3ε for 72 hrs, after which an ELISA was performed on the culture supernatants. 12 corneal allograft acceptor mice were used in 4 separate experiments.

$CD4^+CD25^+$ Tregs express the IL-17 receptor and are potentiated by IL-17A treatment

The previous data demonstrated that IL-17A promoted suppression of $CD4^+$ effector T cell activation, but it was still unclear whether IL-17A directly suppressed the activation of $CD4^+CD25^-$ T effector cells, or if the cytokine promoted $CD4^+CD25^+$ Treg immunosuppressive function. To answer the first question, IL-17 receptor expression was assessed on $CD4^+CD25^+$ $CFSE^-$ Tregs 72 hrs following co-culture with CFSE-labeled $CD4^+$ effector T cells in the presence of anti-CD3ε. Significant levels of the IL-17 receptor protein were detected on the putative Tregs (**Fig. 19A**). Next, we wanted to assess if IL-17A could be used to potentiate naïve $CD4^+CD25^+$ Tregs. To address this, two concentrations of recombinant murine IL-17A (0.5 μg/ml or 1μg/ml) were added to either anti-CD3ε antibody-treated $CD4^+$ T effector cell cultures alone, or naïve $CD4^+CD25^+$ Treg and anti-CD3ε antibody-treated $CD4^+$ T effector cell co-cultures. Suppression of T cell activation was assessed by determining CFSE-labeled naïve $CD4^+$ T effector cell activation. Addition of either 0.5 μg or 1μg of IL-17A to cultures of $CD4^+$ T effector cells alone produced modest suppression (10-12%) (**Fig. 19B**). By contrast, addition of either 0.5 μg or 1μg of IL-17A to co-cultures containing naïve $CD4^+CD25^+$ Tregs and $CD4^+$ T effector cells significantly suppressed effector cell proliferation by 58.4% ± 12.5% and 65.1% ± 12.1% respectively ($p < 0.05$) compared to $CD4^+$ T cells alone (12.2% ± 1.8%, and 10.7% ± 5.8%, respectively).

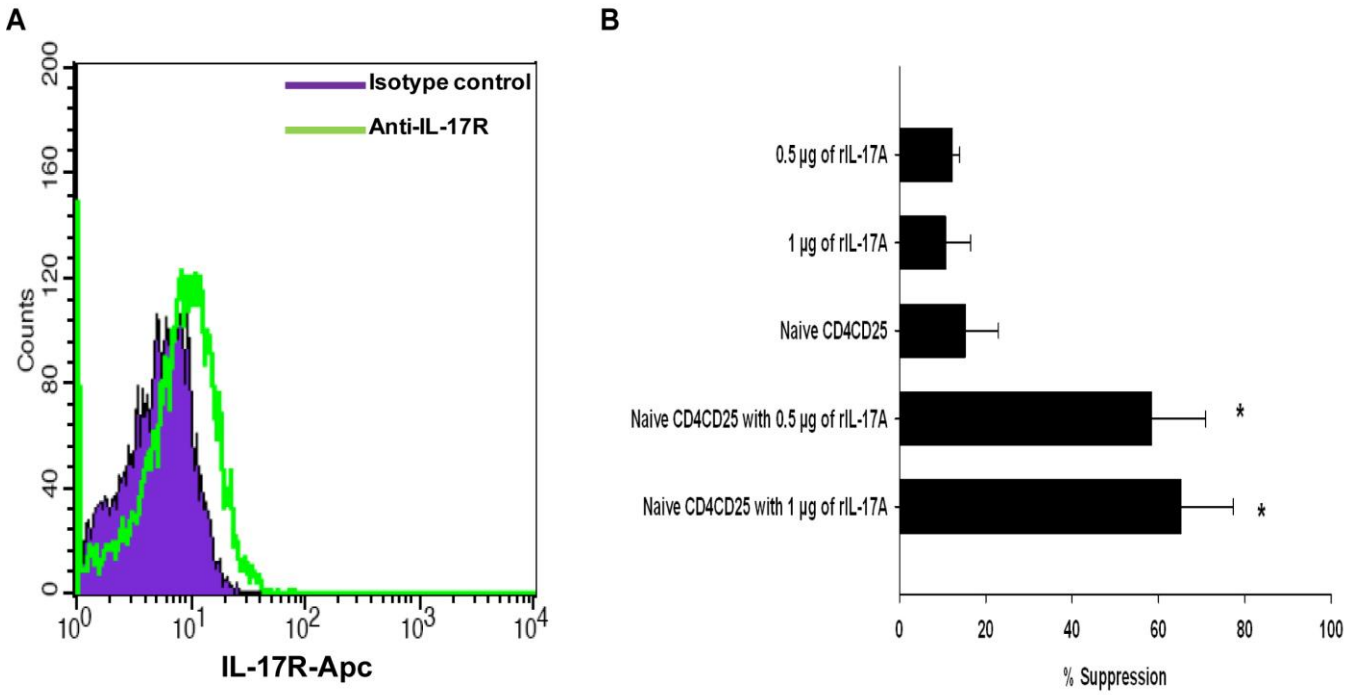

Figure 19. $CD4^+CD25^+$ Tregs express IL-17R and can be potentiated with IL-17A treatment. **A)** Expression of IL-17R was assessed on $CD4^+CD25^+$ $CFSE^-$ Tregs 72 hrs following co-culture with CFSE labeled $CD4^+$ effector T cells in the presence of anti-CD3ε Ab using rat anti-mouse IL-17R Apc. This experiment was performed 2 separate times with similar results using a total of 6 acceptor mice **B)** The ability of exogenous rIL-17A to suppress $CD4^+$ T effector cell activation was determined in an *in vitro* CFSE suppression assay. This experiment was performed 2 separate times with similar results using a total of 8 naïve mice. *$p < 0.05$.

$CD4^+CD25^+$ Treg-mediated suppression is contact-dependent

$CD4^+CD25^+$ Tregs may elicit their suppressive effects by production of soluble immunosuppressive factors and/or by cell-cell contact with effector T cells. *In vitro* transwell culture assays were used to determine if corneal allograft acceptor Treg-mediated suppression was contact-dependent or was mediated through soluble factors. $CD4^+CD25^+$ Tregs were placed in the upper transwell chamber, while $CD4^+$ T effector cells were added to the lower chamber of the transwell culture system that also contained anti-CD3ε antibody. Separation of $CD4^+CD25^+$ Tregs and $CD4^+$ T effector cells with transwell membranes in the co-cultures resulted in an 80% reduction in Treg-mediated suppression compared to co-cultures without a transwell membrane (**Fig. 20A**). These results suggested that corneal acceptor $CD4^+CD25^+$ Treg-mediated suppression was primarily dependent on membrane-bound suppressive molecules. Thus, we examined the expression of several membrane suppressive molecules, notably CTLA-4, GITR, and membrane TGF-β1 on $CD4^+CD25^+$ $CFSE^-$ Tregs 72 hrs following co-culture with CFSE-labeled $CD4^+$ effector T cells in the presence of anti-CD3ε (**Fig. 20B-D**).

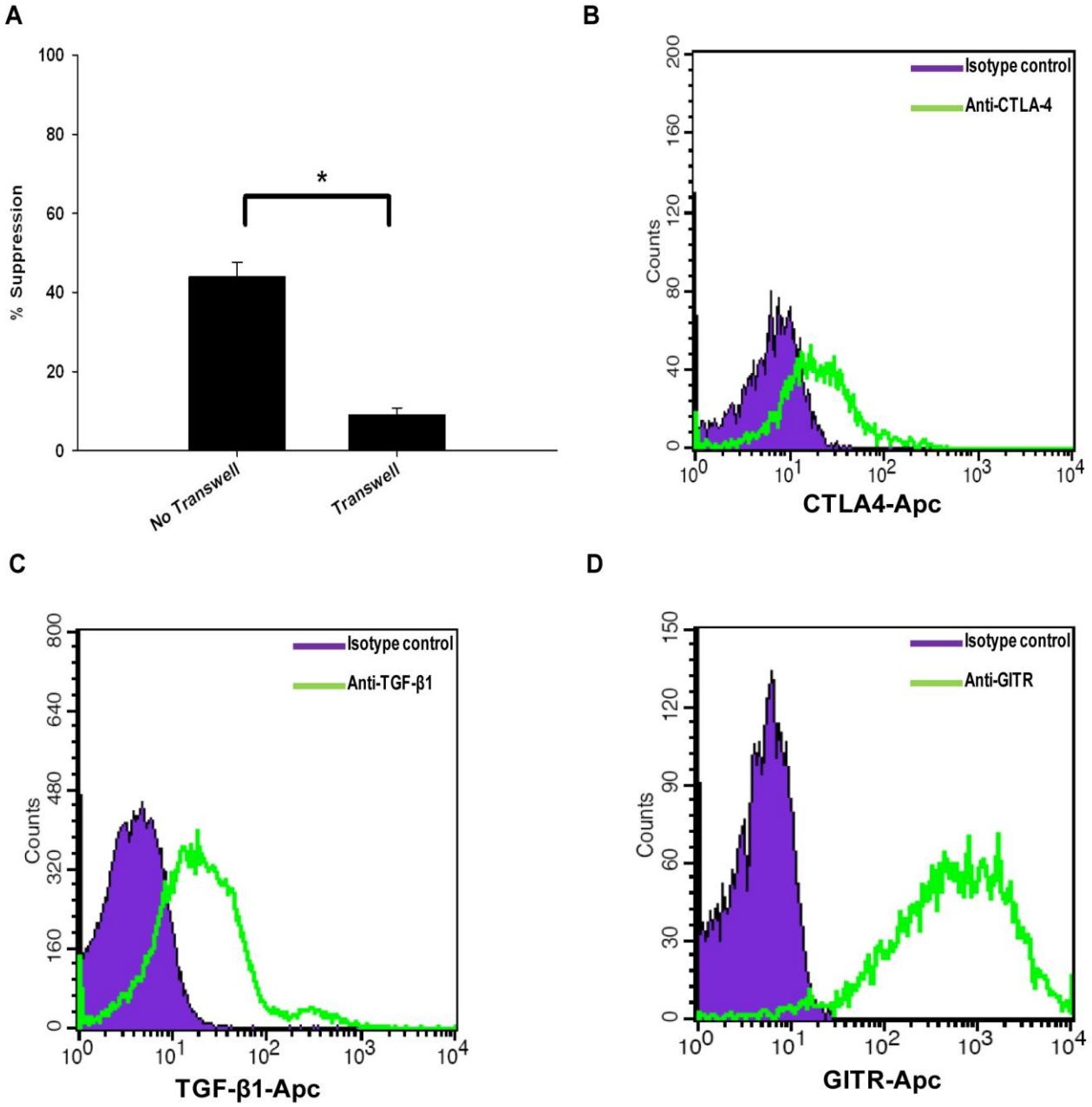

Figure 20. $CD4^+CD25^+$ Treg-mediated suppression is contact dependent. **A)** $CD4^+CD25^+$ Treg contact-dependent suppression was assessed in a transwell culture system. Naïve CFSE-labeled $CD4^+$ T effector cells were stimulated with anti-CD3ε Ab for 72 hrs as described above. $CD4^+CD25^+$ T cells from corneal allograft acceptor mice were either co-cultured with $CD4^+$ T effector cells or added to the top chamber of transwell culture plates and $CD4^+$ T effector cells added to the bottom chamber. A 0.4-μm pore sized membrane separated the two chambers. Three independent experiments were performed using $CD4^+CD25^+$ Tregs from a total of 9 corneal allograft acceptor mice yielded similar results (*$p < 0.05$). Using Apc-conjugated Abs, expression of **B)** CTLA-4, **C)** mTGF-β1 and **D)** GITR was assessed on $CD4^+CD25^+$ $CFSE^-$ Tregs 72 hrs following co-culture with CFSE labeled $CD4^+$ effector T cells in the presence of anti-CD3ε Ab. This experiment was performed 2 separate times with similar results using a total of 6 acceptor mice**.**

Depletion of IL-17A impairs CD4$^+$CD25$^+$ Treg contact-mediated suppressive potential

The experiments described above demonstrated that IL-17A is required for CD4$^+$CD25$^+$ Treg suppressive activity (**Fig. 17A**). Accordingly, *in vitro* assays were used to test the hypothesis that depletion of IL-17A downregulated the expression of cell membrane-bound immunosuppressive molecules. CD4$^+$CD25$^+$ Tregs were stimulated with anti-CD3ε antibody and co-cultured for 72 hrs with naïve CFSE-labeled CD4$^+$ effector T cells in the presence of either anti-IL-17A or an isotype control antibody. CFSE-negative cells (CD4$^+$CD25$^+$ Tregs) were subsequently sorted and used for total RNA isolation. Quantitative PCR analysis of CD4$^+$CD25$^+$ Treg RNA showed that exposure to anti-IL-17A antibody resulted in a ≥ 2 fold decrease in the expression of FoxP3, and three membrane-bound suppressive molecules, namely CTLA-4, GITR, and TGF-β1 (**Fig. 21A**). To confirm that CD4$^+$CD25$^+$ Tregs mediated the inhibition of CD4$^+$ effector T cell activation through one or more of the membrane-bound suppressive molecules, blocking antibodies (anti-CTLA-4, anti-GITR-L, and anti-TGF-β1) were added to co-culture assays containing CD4$^+$CD25$^+$ Tregs and CD4$^+$ T effector cells. When used independently, the antibodies did not cause a significant decrease in suppression when compared to the isotype controls. By contrast, simultaneous blockade of both GITR and CTLA-4 led to a significant decrease in suppression ($p < 0.05$), and the most dramatic loss of suppression came with the addition of all three blocking antibodies, which inhibited approximately 50% of the suppression mediated by CD4$^+$CD25$^+$ Tregs (**Fig. 21B**). To our knowledge, this is the first evidence that IL-17A plays a crucial role in the maintenance of CD4$^+$CD25$^+$ Treg contact-mediated suppression.

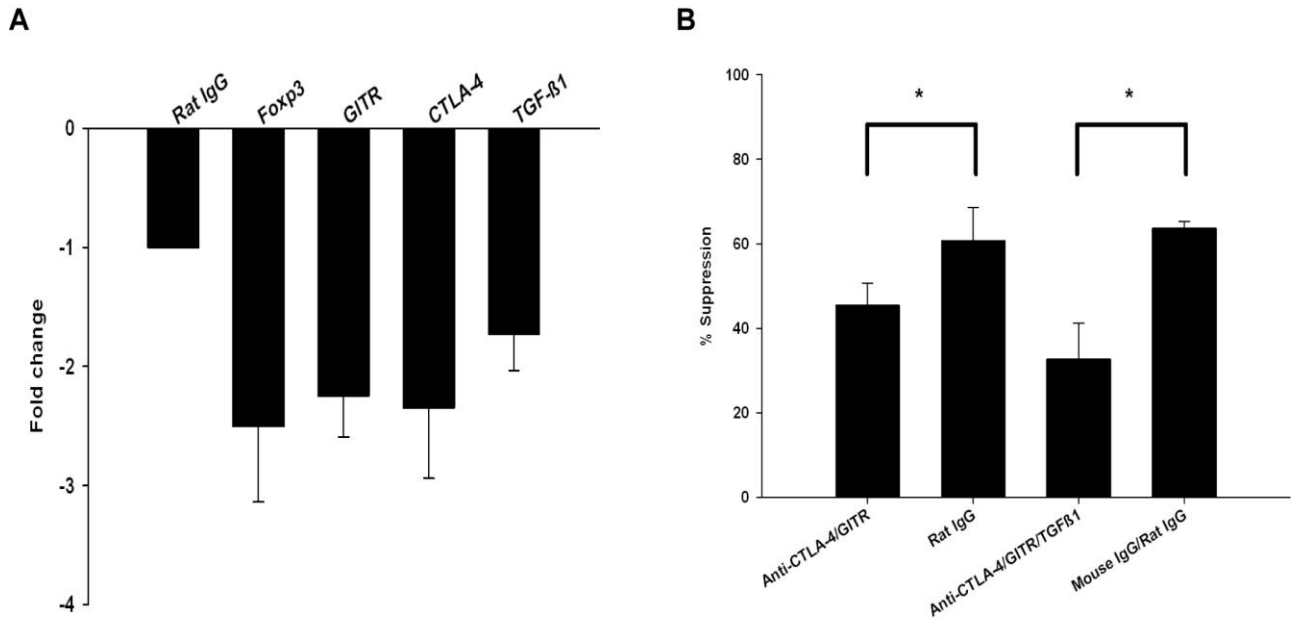

Figure 21. Depletion of IL-17A impairs $CD4^+CD25^+$ Treg contact-mediated suppressive potential. A) Effect of anti-IL-17A on the expression of cell membrane-bound suppressive molecules was determined by culturing corneal allograft-induced $CD4^+CD25^+$ Tregs with CFSE-labeled naïve $CD4^+$ T cells for 72hrs in the presence of either anti-IL-17A or an isotype IgG control antibody. $CD4^+CD25^+$ Tregs were separated from the CFSE-labeled naïve $CD4^+$ T cells by cell sorting. $CD4^+CD25^+$ Treg expression of mRNA for Foxp3, GITR, CTLA-4, and TGF-β1 was assessed by qPCR. $p < 0.05$ for all 4 groups compared to the rat IgG control group. Three independent experiments using a total of 9 corneal allograft acceptor mice were performed with similar results. **B)** Antibody blockade of membrane molecules significantly curtailed $CD4^+CD25^+$ Treg-mediated suppression. $CD4^+CD25^+$ Tregs and naïve CFSE-labeled $CD4^+$ T effector cells co-cultures were incubated in the presence of various combinations of anti-CTLA-4, anti-GITRL, and anti-TGFβ1 antibodies at concentrations of 10 µg/ml, 10µg/ml, and 50µg/ml respectively and suppression mediated by $CD4^+CD25^+$ Tregs was assessed by *in vitro* CFSE-based immunosuppression assay. $*p < 0.05$. Two independent experiments were performed using a total of 12 corneal allograft acceptor mice which yielded similar results.

Acceptor $CD4^+CD25^+$ Tregs are efferent suppressors and are antigen-specific

We investigated whether the $CD4^+CD25^+$ Tregs suppressed the efferent phase of corneal alloantigen immune responses by inhibiting allogeneic DTH. To test for efferent immune suppression, a local adoptive transfer (LAT) assay was employed in which $CD4^+CD25^+$ T cells were isolated from corneal allograft rejector and acceptor mice 3 weeks post transplantation. $CD4^+CD25^+$ T cells were also collected from naïve mice as controls. $CD4^+$ T effector cells were isolated from corneal allograft rejector mice. APCs from naïve BALB/c mice were pulsed with C57BL/6 antigens, combined with $CD4^+CD25^+$ T cells and $CD4^+$ T effector cells in a 1:1:1 ratio and injected into the ear pinnae of naïve BALB/c mice. $CD4^+CD25^+$ T cells from either naïve or corneal allograft rejector mice did not suppress DTH responses. By contrast, $CD4^+CD25^+$ T cells from corneal allograft acceptor mice significantly inhibited ear swelling, thereby supporting the hypothesis that $CD4^+CD25^+$ Tregs suppress the efferent phase of the alloimmune response (**Fig. 22A**). To assess whether the efferent suppression was antigen-specific, LAT assays were performed using naïve BALB/c APCs pulsed with either C3H or C57BL/6 spleen cell lysates and incubated with $CD4^+$ T effector cells isolated from BALB/c mice that had rejected either C3H or C57BL/6 corneal allografts, respectively. $CD4^+CD25^+$ Tregs from BALB/c mice that accepted C57BL/6 allografts were added in equal proportions to the cell mixtures and injected into the ear pinnae of naïve BALB/c mice. $CD4^+CD25^+$ Tregs from BALB/c mice that accepted C57BL/6 corneal allografts inhibited DTH responses to C57BL/6 alloantigens, but had no effect on the DTH response to C3H alloantigens, thus confirming alloantigen specificity of the corneal allograft-induced $CD4^+CD25^+$ Tregs (**Fig. 22B**).

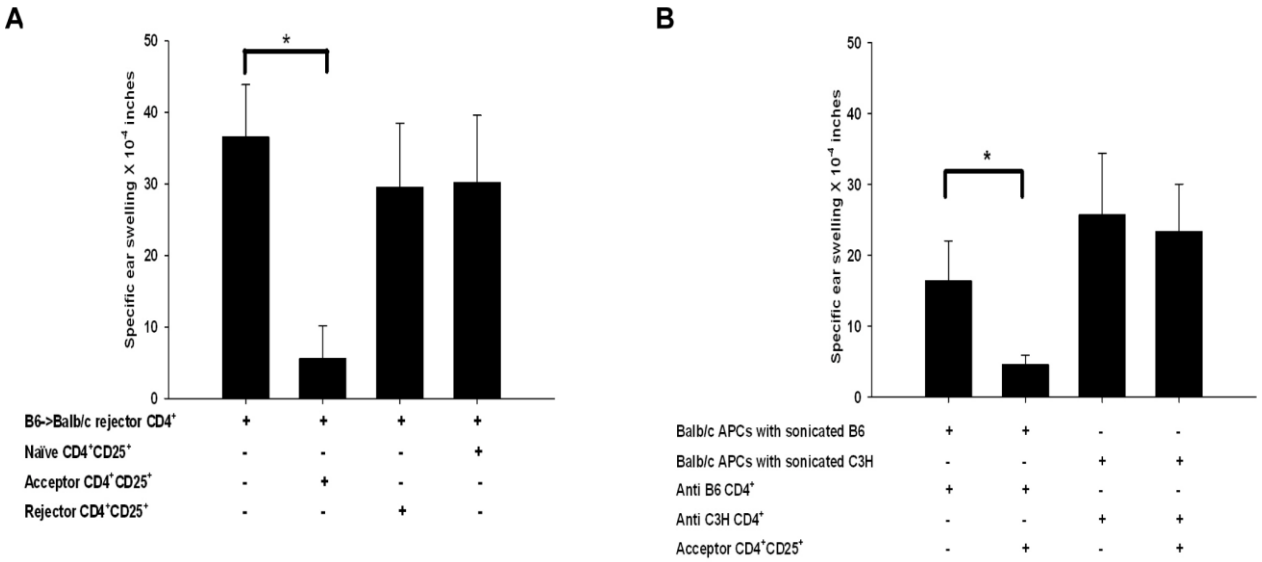

Figure 22. Corneal allograft-induced $CD4^+CD25^+$ Tregs suppress the efferent arm of the immune response and are antigen-specific. A) Naïve $CD4^+CD25^+$ and corneal allograft-induced $CD4^+CD25^+$ Tregs from corneal acceptor and rejector mice were mixed with $CD4^+$ T cells from corneal allograft rejector mice and APCs pulsed with C57BL/6 alloantigens and used in a LAT assay for DTH ear swelling assays. *$p < 0.05$. N = 5 per group. Two independent experiments were performed with similar results. **B)** C57BL/6 corneal allograft-induced $CD4^+CD25^+$ Tregs from BALB/c hosts were mixed with $CD4^+$ T cells from either BALB/c mice that rejected C57BL/6 corneal allografts or BALB/c mice that rejected a C3H corneal allograft. APCs pulsed with either C57BL/6 or C3H alloantigens were admixed with the aforementioned T cell suspensions and injected into the ear pinnae of naïve BALB/c mice. Ear swelling was measured 24 hrs later. *$p < 0.05$. This experiment was performed two times with similar results. N = 5 per group.

Acceptor $CD4^+CD25^+$ Tregs act transiently

The ability of IL-17A-activated $CD4^+CD25^+$ Tregs to suppress the efferent arm of the immune response suggests that hosts with long-term surviving corneal allografts possess Tregs that persist indefinitely and promote the survival of subsequent corneal allografts. To determine if $CD4^+CD25^+$ Tregs are required for long-term survival of corneal allografts, BALB/c mice bearing clear C57BL/6 allografts for 30 days were treated with either anti-IL-17A plus anti-CD25 antibodies, or isotype control antibodies twice a week for 60 days. Interestingly, combined treatment with anti-IL-17A and anti-CD25 antibodies did not significantly affect the survival of corneal allografts that had been clear for 30 days compared to mice treated with isotype control antibodies (**Fig. 23**). These results indicate that the $CD4^+CD25^+$ Treg-mediated suppression is necessary for corneal allograft survival during the initial sensitization phase of the alloimmune response, but is superfluous 30 days post transplantation.

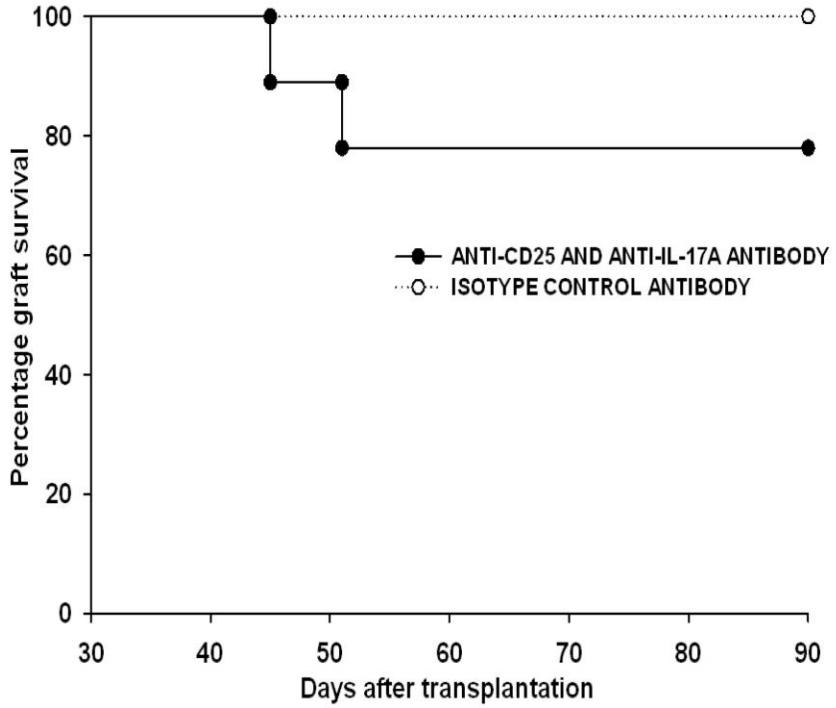

Figure 23. $CD4^+CD25^+$ Treg suppression is not required for long-term corneal allograft survival and is transient. BALB/c recipients with clear C57BL/6 corneal allografts at day 30 were treated with either a combination of anti-CD25 and anti-IL-17A (N = 9) or an isotype control antibody (N = 7) twice per week for 60 days. Corneal allografts in both groups of mice had MSTs of 90 days. There was no significant difference in the incidence of graft rejection between the two groups ($p > 0.05$).

Acceptor $CD4^+CD25^+$ Tregs are transient

Although $CD4^+CD25^+$ Tregs are not required to maintain long-term corneal allograft survival as shown above, they may be necessary for survival of subsequent allograft transplants in mice that have accepted an initial corneal transplant. Accordingly, BALB/c mice that successfully accepted C57BL/6 corneal allografts were given a second C57BL/6 corneal allograft to the contralateral eye 90 days after the initial allograft transplant and administered bi-weekly treatments with anti-IL-17A and anti-CD25 antibodies, or isotype control antibody. Second corneal allografts on the anti-IL-17A-treated and anti-CD25-treated mice underwent rejection in 100% of the hosts, compared to primary allografts, which exhibited a 50% rejection (**Fig. 24A**). Moreover, 86% of the isotype-treated mice rejected their second corneal allografts grafts compared to the 50% rejection of the primary corneal allografts (**Fig. 24B**). There was no significant difference in the MSTs for the second corneal allografts for the anti-IL-17A and anti-CD25-treated group (10 days) and for the isotype control-treated animals (7 days) ($p > 0.05$). However, there were significant differences in the tempos of rejection for the second corneal allografts with either treatment compared to the primary grafts ($p < 0.05$). Together, these results suggest that memory $CD4^+CD25^+$ Tregs were either absent, or the existence of Tregs in hosts that accept corneal allografts is transient.

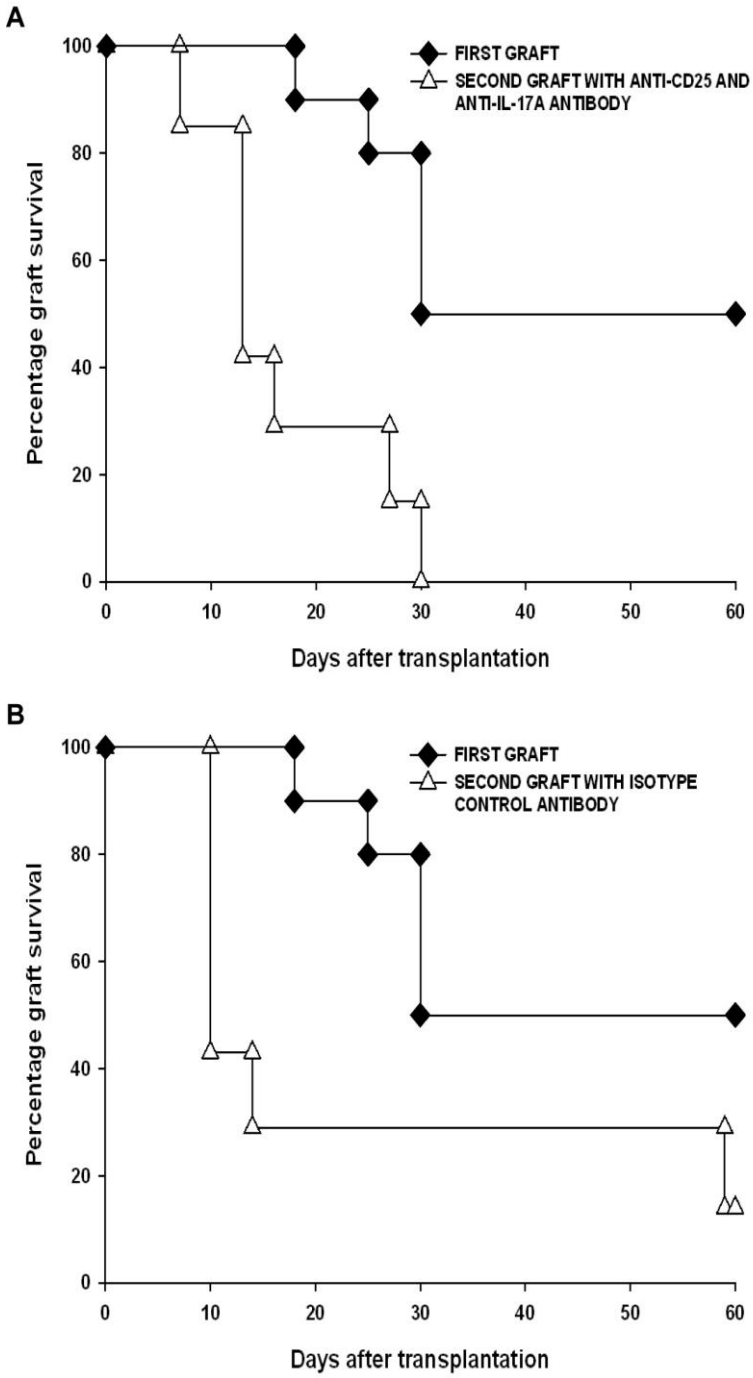

Figure 24. $CD4^+CD25^+$ Tregs are not long-lasting. BALB/c mice with clear primary C57BL/6 corneal allografts 90 days post transplantation received a second C57BL/6 corneal allograft applied to the contralateral eye and received continued treatment with a combination of **A)** anti-CD25 and anti-IL-17A antibodies or **B)** isotype control antibody given twice per week for the duration of the experiment. The MSTs for the second grafted corneas were 10 days for the anti-CD25 and anti-IL-17A-treated group and 7 days for the isotype control-treated animals (N = 7 per group). The MST for the primary corneal allograft was 46 days (N = 10). $p < 0.05$ by Kaplan-Meier survival analysis for the second grafted corneas with either treatment compared to primary grafts.

Systemic administration of rIL-17A does not increase corneal immune privilege

Previous reports had indicated that systemic administration of IL-17A mitigated the severity of the several diseases including uveitis and colitis (72, 73, 75, 187). Thus, we similarly administered recombinant murine IL-17A (rIL-17A) to mice on days -2, -1 prior to, and every other day post corneal allograft transplantation. Control mice received PBS only. Two doses of rIL-17A were used for I.P. injections. For the first experiment using 0.3μg injections of rIL-17A, 60% of the mice had surviving corneal allograft after 60 days compared to 30% in the PBS-treated mice (**Fig. 25A**). The rIL-17A-treated animals had an MST of 60 days while the MST in the PBS-treated animals was 33 days. However, no significant difference in the tempo of allograft rejection between the two groups was found by log-rank analysis. At the 0.5μg dose, the incidence of graft survival was 70% in the rIL-17A-treated group with an MST of 60 days compared to 55% graft survival in the PBS control group with an MST of 60 days (**Fig. 25B**). Like the mice receiving the 0.3μg injections, no significant difference in the tempo of graft rejection was observed between the 0.5μg IL-17A-treated group and the PBS control group.

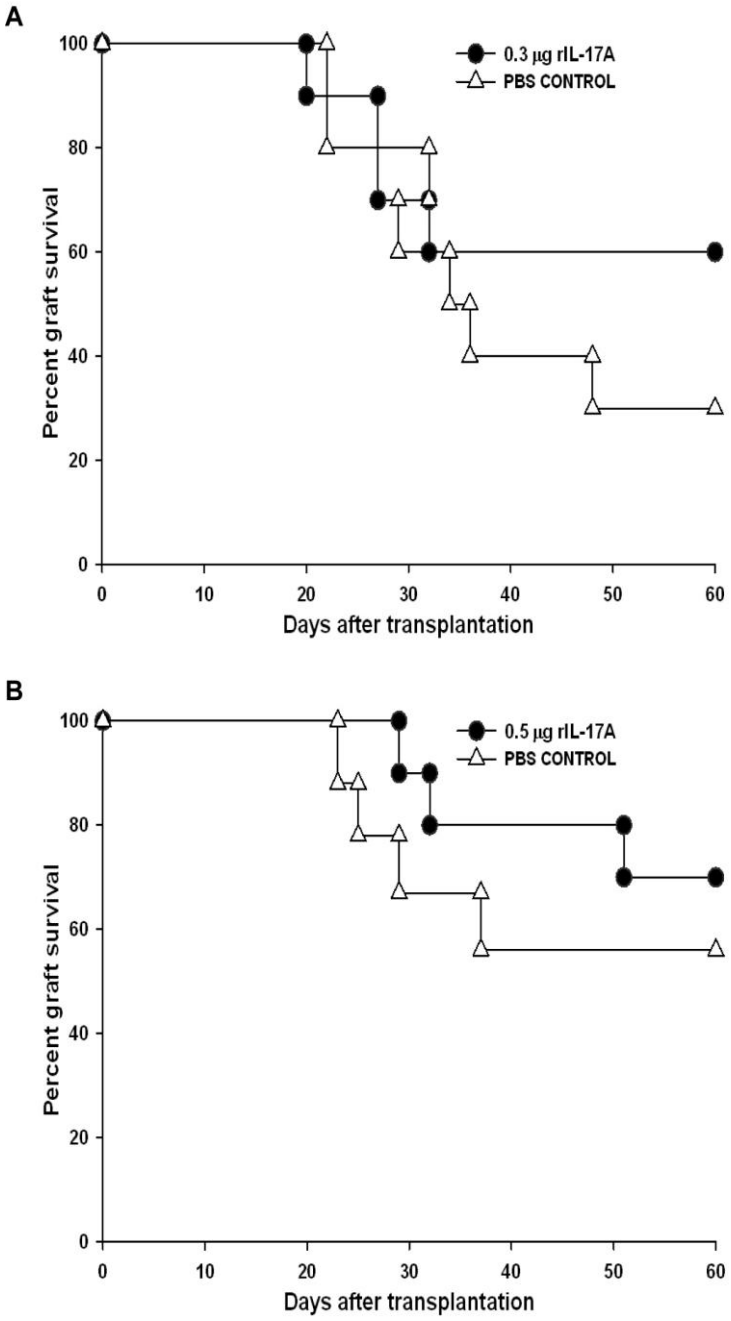

Figure 25. Systemic administration of rIL-17A does not enhance corneal allograft survival. A) BALB/c mice were treated with I.P. injections of either 0.3 µg of rIL-17A or PBS on days -2, -1, 0, and every other day following C57BL/6 corneal allograft transplantation. C57BL/6 corneal allografts underwent rejection in 30% of the rIL-17A-treated BALB/c mice (N = 10) and had an MST of 60 days compared to the 70% rejection in the PBS-treated group (N = 10) which had an MST of 33 days ($p > 0.05$). **B)** BALB/c mice were treated with either 0.5 µg of rIL-17A or PBS on days -2, -1, 0, and every other day following C57BL/6 corneal allograft transplantation. C57BL/6 corneal allografts underwent rejection in 30% of the rIL-17A-treated BALB/c mice (N = 10) and had an MST of 60 days compared to the 45% rejection in the PBS-treated group (N = 10) which had an MST of 60 days ($p > 0.05$).

ROLE OF ACAID IN CORNEAL IMMUNE PRIVILEGE

ACAID is an aberrant systemic immune response induced when certain antigens are introduced into the AC (99). ACAID leads to the active suppression of DTH responses, which is also observed in hosts bearing long-term corneal allografts (132). Due to the juxtaposition of corneal allografts to the AC of the eye, it is commonly thought that the shedding of corneal cells into this AC compartment following keratoplasty would lead to the induction of ACAID. Indeed, procedures such as splenectomy, which inhibit the induction of ACAID, have also been shown to abolish the immune privilege of corneal allografts (121, 134, 135). Moreover, several reports in both mouse and rat models of keratoplasty have indicated that introduction of alloantigens into the AC of hosts significantly reduces graft rejection by up to 65% (34, 35). Accordingly, the requirement for IL-17A-dependent $CD4^+CD25^+$ ACAID Tregs was examined.

CD25$^+$ Tregs are required for corneal allograft survival and ACAID

Previous studies have shown that the induction of ACAID requires the participation of two independent Treg populations (99). One population is $CD4^+$ and acts at the afferent arm to prevent allosensitization, while the other Treg population is $CD8^+$ and acts at the efferent arm of the immune response to suppress DTH responses produced by previously sensitized T cells (188). Since many $CD4^+$ Tregs also express CD25, we wished to determine if *in vivo* treatment with a blocking anti-CD25 antibody would affect ACAID and corneal allograft survival. Mice were treated with either anti-CD25 antibody or an isotype control antibody one day before and at weekly intervals after administering either an AC injection with C57BL/6 spleen cells or an orthotopic corneal allograft. As described previously, anti-CD25 treatment robbed the corneal allograft of its immune privilege (**Fig. 26A**). Mice injected in the AC with C57BL/6 spleen cells were immunized SC with C57BL/6 spleen cells seven days later and DTH was evaluated seven days after the SC immunization. The results indicated that *in vivo* treatment with anti-CD25 antibody prevented the development of ACAID (**Fig. 26B**).

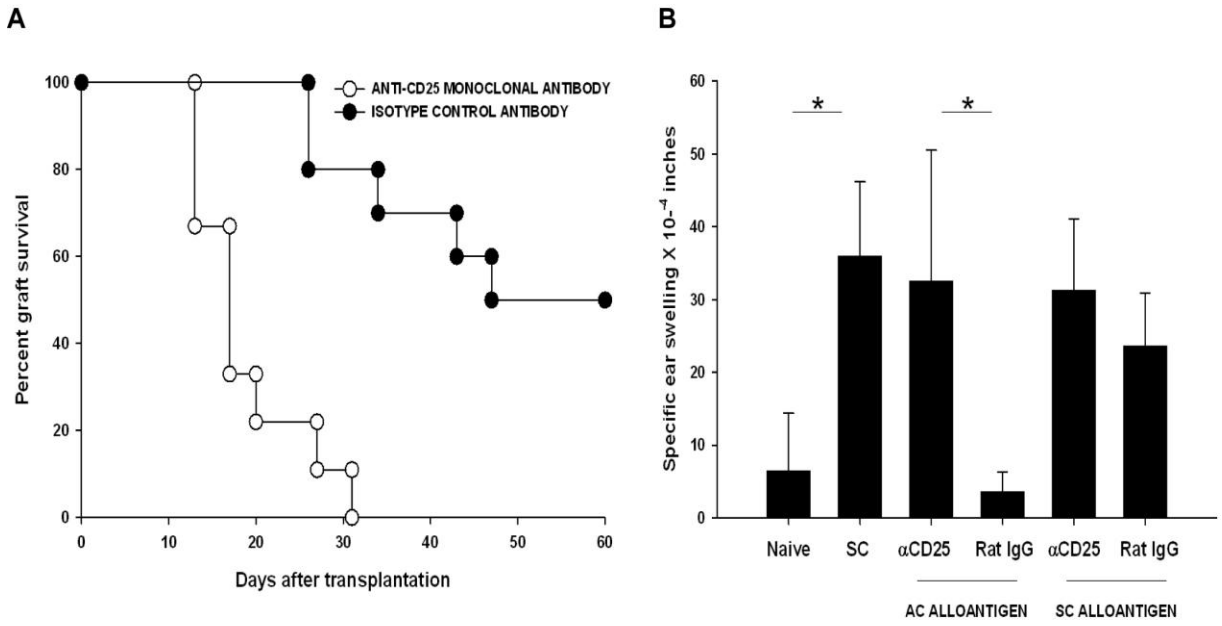

Figure 26. CD4$^+$CD25$^+$ Tregs are required for corneal allograft survival and ACAID. **A)** BALB/c mice were treated with 500 μg of anti-CD25 antibody or isotype control once prior to and once weekly following corneal transplantation or AC injection. C57BL/6 corneal allografts underwent rejection in 50% of hosts treated with the isotype control IgG (N = 10) and had an MST of 52 days. C57BL/6 corneal allografts transplanted to BALB/c recipients treated with anti-CD25 were rejected in 100% of hosts with an MST of 26 days respectively (N = 10). $p < 0.05$ between the anti-CD25 treated group and rat IgG isotype control-treated allograft recipients. The experiment was performed twice with similar results. **B)** ACAID was induced on day 0 with C57BL/6 splenic non-adherent cells. A SC injection of C57BL/6 splenocytes was given on day +7. DTH challenge with mitomycin-C-treated C57BL/6 cells was given on day +14. Negative control animals received an ear challenge only. N = 5 for all groups (*$p < 0.05$). This experiment was performed twice with similar results.

Cyclophosphamide abolishes corneal immune privilege and ACAID

It has been reported that low-dose cyclophosphamide inhibits the activity of $CD4^+CD25^+$ Tregs without producing global immunosuppression (176). Accordingly, mice were treated with IP injections of cyclophosphamide (100 mg/injection) one day before either AC injection of C57BL/6 spleen cells or orthotopic transplantation of C57BL/6 corneal allografts and at 7-day intervals thereafter. Mice injected in the AC with C57BL/6 spleen cells were immunized SC with C57BL/6 spleen cells seven days later and DTH was evaluated seven days after the SC immunization. Low-dose cyclophosphamide treatment accelerated rejection of C57BL/6 corneal allografts (**Fig. 27A**) and prevented the development of ACAID (**Fig. 27B**). Cyclophosphamide treatment alone did not produce an adjuvant effect nor did it enhance the baseline DTH response in SC immunized mice, as the responses in SC immunized mice without cyclophosphamide treatment were identical to the responses of cyclophosphamide-treated mice that were immunized SC with C57BL/6 spleen cells (**Fig. 27B**). The effect of cyclophosphamide in the increased incidence of corneal allograft rejection was not due to a toxic effect, as BALB/c mice treated with cyclophosphamide did not reject syngeneic BALB/c corneal homografts (**Fig. 27A**).

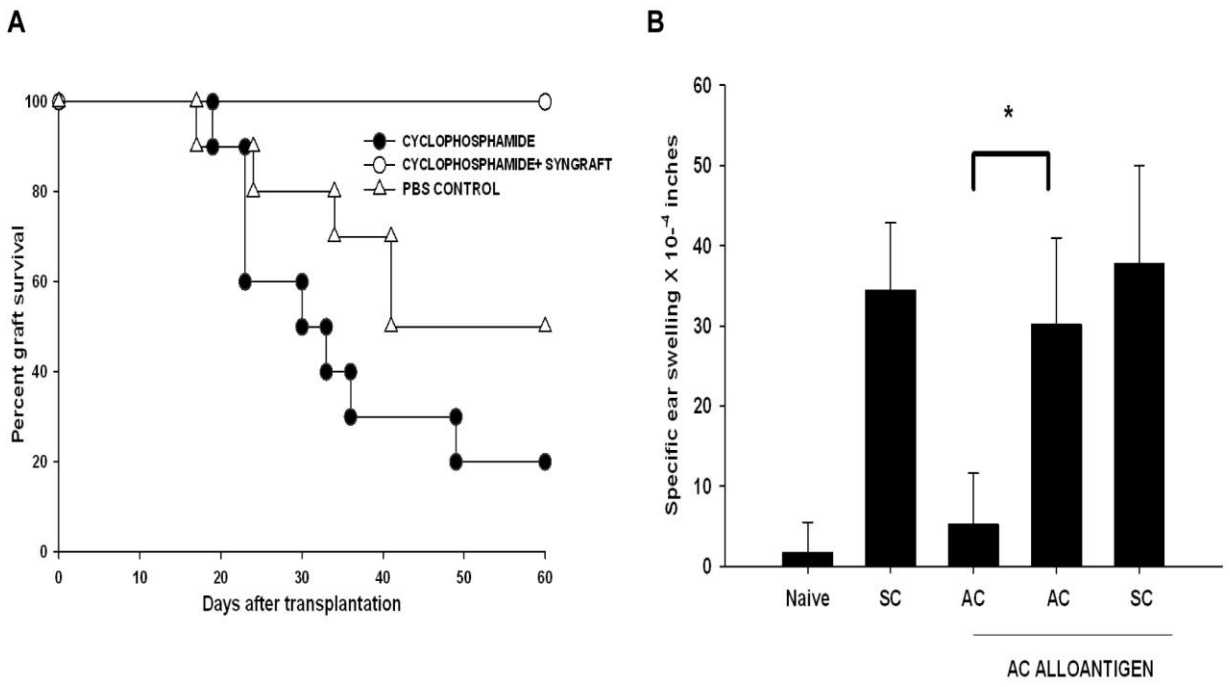

Figure 27. Cyclophosphamide abolishes ACAID and corneal immune privilege. Cyclophosphamide treatments were performed one day prior to and once per week following AC injection or corneal transplantation. **A)** C57BL/6 corneal allograft survival in BALB/c mice treated with cyclophosphamide. C57BL/6 corneal allografts underwent rejection in 50% of untreated recipients (N = 10) and had an MST of 52 days. BALB/c hosts treated with cyclophosphamide rejected 80% of grafts with an MST of 28 days (N = 10). $p < 0.05$ between cyclophosphamide-treated and untreated allograft recipients. No rejection was observed in the syngeneic recipient group treated with cyclophosphamide. **B)** ACAID was induced on day 0 by AC injection of C57BL/6 splenic non-adherent cells. A SC injection of C57BL/6 splenocytes was given on day +7. DTH challenge with mitomycin-C-treated C57BL/6 cells was administered on day +14. Negative control animals received an ear challenge only. Positive and DTH control animals were immunized SC and received an ear challenge, but were not injected in the AC. *p = 0.001 for cyclophosphamide-treated vs untreated group in which ACAID was induced (N = 5). The experiment was performed twice with similar results.

IL-17A is required for corneal allograft survival but not ACAID

We further examined the effect of *in vivo* neutralization of IL-17A on the induction of ACAID and the immune privilege of corneal allografts. Mice were treated with either monoclonal anti-IL-17A or an isotype control antibody on days -4 and -2 prior to either AC injection of C57BL/6 non-adherent spleen cells or the application of an orthotopic C57BL/6 corneal allograft and twice weekly thereafter. As shown previously, systemic administration of anti-IL-17A abolished corneal immune privilege (**Fig. 28A**). By contrast, in each of several repeated experiments, we found that administration of this same monoclonal antibody did not affect the development of ACAID (**Fig. 28B**). Although IL-17A is not necessary for the generation of one form of ocular immune privilege (i.e., ACAID), it is absolutely required for the immune privilege of corneal allografts. Not only did neutralization of IL-17A abolish the immune privilege of corneal allografts, but it also led to accelerated graft rejection.

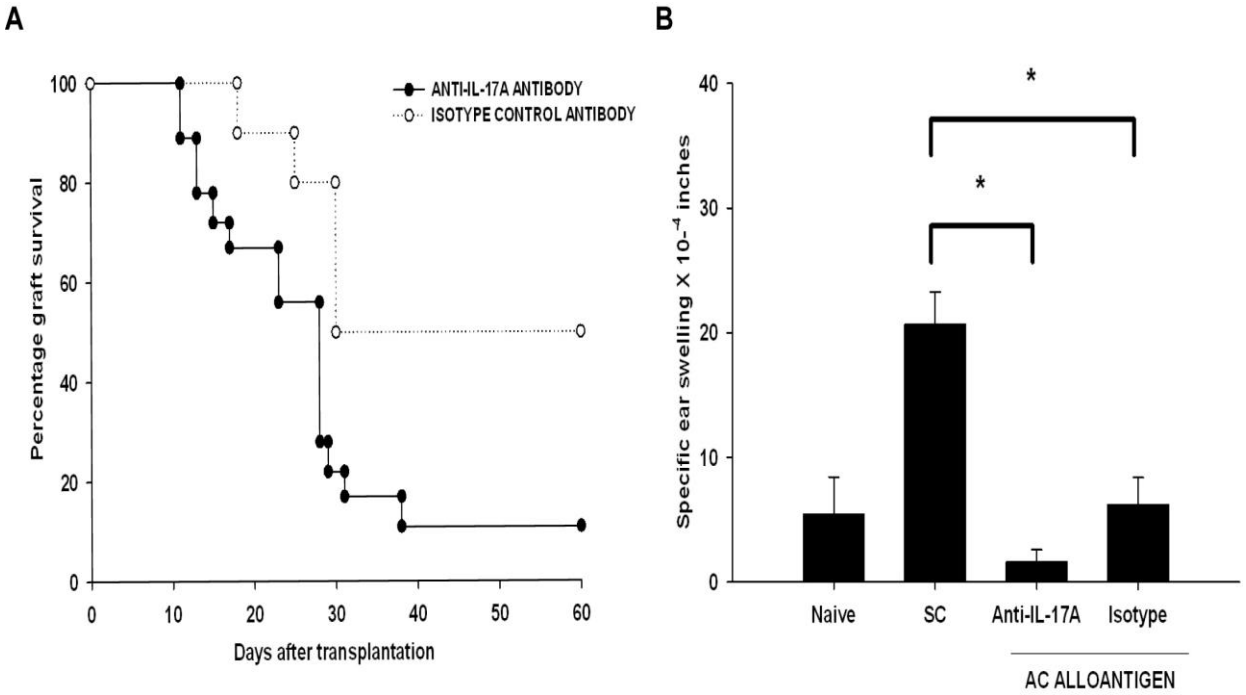

Figure 28. IL-17A is required for corneal allograft survival but not ACAID. BALB/c mice were treated with anti-IL-17A antibody or isotype control on days -4 and -2 prior to and twice weekly following corneal transplantation or AC injection. **A)** C57BL/6 corneal allograft survival in BALB/c mice treated with anti-IL-17A or a rat IgG isotype control antibody. C57BL/6 corneal allografts underwent rejection in 50% of hosts treated with the isotype control IgG (N = 10) and had an MST of 46 days. C57BL/6 corneal allografts transplanted to BALB/c recipients treated with anti-IL-17A were rejected in 90% of hosts with an MST of 14.5 days respectively (N = 10). $p < 0.05$ between anti-IL-17A-treated group and rat IgG isotype control treated allograft recipients. The experiment was performed four times with similar results. **B)** ACAID was induced on day 0 with C57BL/6 splenic non-adherent cells. SC injections of C57BL/6 splenocytes were given on day +7. DTH challenge with mitomycin-C-treated C57BL/6 cells was on day +14. Negative control animals received an ear challenge only. ACAID groups treated with anti-IL-17A or isotype control antibody were injected in the AC with C57BL/6 antigen, SC immunized, and ear challenged with mitomycin-C-treated C57BL/6 splenocytes. *$p < 0.05$ for the anti-IL-17A-treated and the isotype control-treated groups vs the SC immunized mice. $p > 0.05$ for the anti-IL-17A-treated vs isotype control-treated group (N = 5). This experiment was performed twice with similar results.

IFN-γ is required for corneal allograft survival and ACAID

It was previously reported that lymph node (LN) cells from mice with OVA-induced ACAID produced significantly less IFN-γ but significantly more IL-4 and IL-10 compared to LN cells from mice that were immunized SC with OVA (129). This was interpreted by some investigators to be evidence that ACAID was the result of Th2 cross-regulation of Th1 responses. We have previously shown that corneal allografts undergo immune rejection in IFN-γ KO mice and in normal mice treated with anti-IFN-γ antibody (171). Since the long-term survival of corneal allografts correlates with the development of Tregs (85), we examined the effect of IFN-γ depletion on the development of ACAID and the fate of corneal allografts. For corneal allograft survival experiments, mice were treated with either anti-IFN-γ antibody or an isotype control antibody on days -4 and -2 before corneal transplantation and twice weekly thereafter. For ACAID experiments, mice were treated with either anti-IFN-γ antibody or an isotype control antibody on days -1 and +7 relative to AC injection with C57BL/6 spleen cells (day 0). Mice injected in the AC with C57BL/6 non-adherent spleen cells were immunized SC with C57BL/6 spleen cells seven days later, and DTH was evaluated seven days after the SC immunization. In agreement with previous experiments, depletion of IFN-γ led to accelerated corneal allograft rejection (**Fig. 29A**). Similarly, mice treated with anti-IFN-γ antibody failed to develop ACAID (**Fig. 29B**). Thus, both ACAID and corneal allograft survival require the presence of IFN-γ.

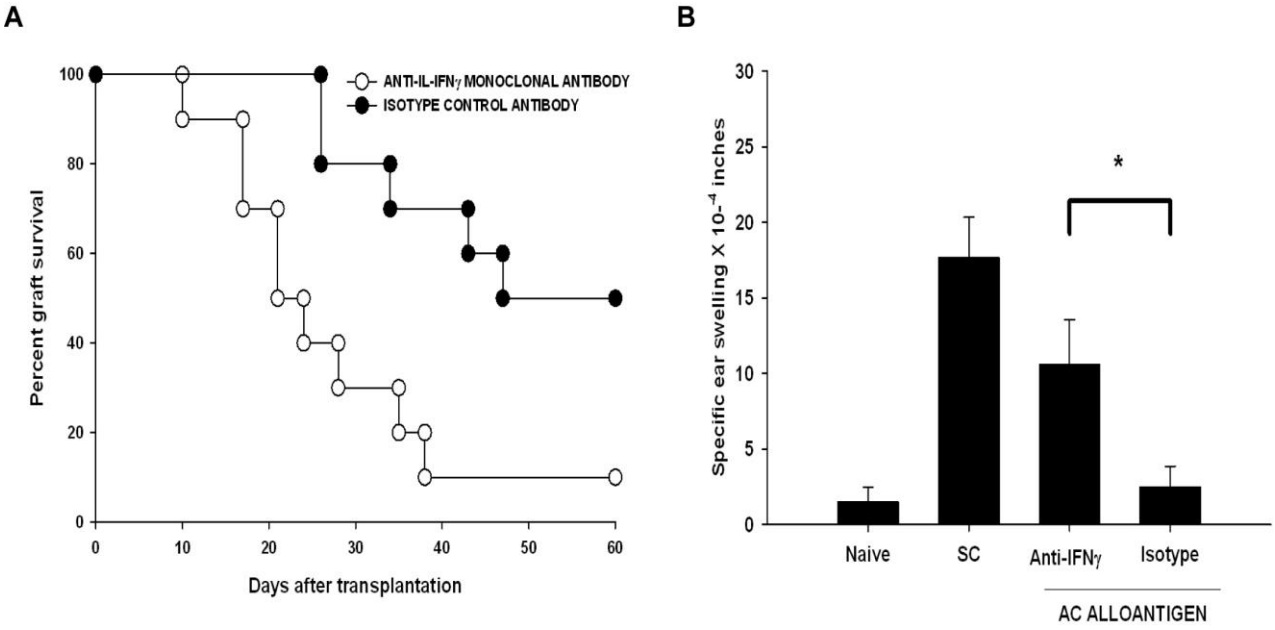

Figure 29. IFN-γ is required for ACAID and corneal immune privilege. **A)** BALB/c animals were treated with anti-IFN-γ antibody or isotype control on days -4 and -2 prior to and twice weekly following corneal transplantation. 90% of BALB/c hosts treated with anti-IFN-γ rejected their C57BL/6 corneal allografts with an MST of 19 days (N = 10). The incidence of rejection of C57BL/6 corneal allografts in BALB/c mice treated with isotype control was 50% (N = 10) and with an MST of 46 days ($p < 0.05$). **B)** Mice were treated with 500μg anti-IFN-γ or isotype control antibody on days -1 and +7. ACAID was induced with C57BL/6 non-adherent spleen cells on day 0, followed by SC immunization with C57BL/6 splenocytes on day +7. DTH challenge was performed with mitomycin-C-treated C57BL/6 cells on day 14. *$p = 0.002$ between anti-IFN-γ and isotype control (N = 5). This experiment was performed 2 additional times with similar results.

CD8$^+$ T cells are required for ACAID but not for the immune privilege of corneal allografts

As stated earlier, a population of CD8$^+$ Tregs is needed for the efferent suppression of DTH responses during ACAID (189, 190). Experiments were performed to determine if *in vivo* administration of anti-CD8 monoclonal antibody would influence the development of ACAID and affect the fate of corneal allografts. Mice were treated with either anti-CD8 antibody or an isotype control antibody on days -4 and -3 and at weekly intervals after either AC injection (day 0) with C57BL/6 spleen cells or orthotopic corneal transplantation. Mice injected in the AC with C57BL/6 spleen cells were immunized SC with C57BL/6 spleen cells seven days later, and DTH was evaluated seven days after the SC immunization. Although anti-CD8 antibody treatment prevented the expression of ACAID (**Fig. 30B**), it did not affect the immune privilege of corneal allografts (**Fig. 30A**). The tempo and incidence of corneal allograft rejection were virtually identical in the anti-CD8 treated mice and the untreated controls (MST= 52 days and 46 days respectively; 60% rejection and 50% rejection respectively; $p > 0.05$).

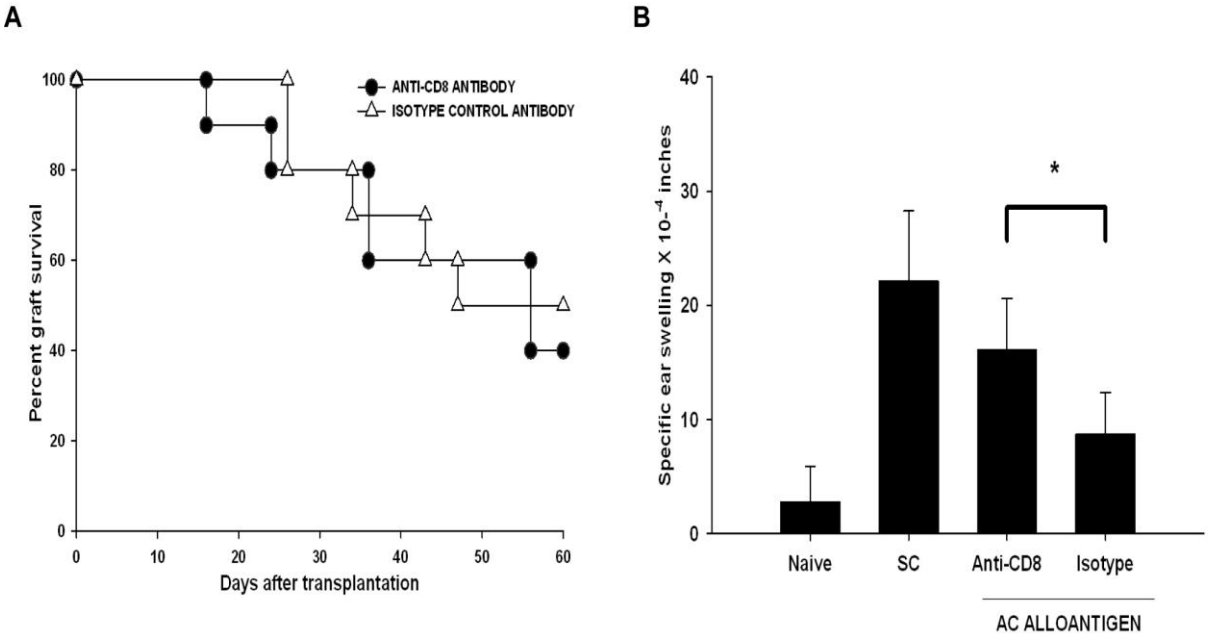

Figure 30. CD8$^+$ T cells are required for ACAID but not corneal allograft survival. **A)** BALB/c hosts were treated with 500 μg anti-CD8 one week prior to and once per week following allograft transplantation. 60% of BALB/c hosts treated with anti-CD8 rejected their C57BL/6 corneal allografts with an MST of 52 days (N = 10). The incidence of C57BL/6 corneal allograft rejection in BALB/c isotype control-treated mice was 50% (N = 10), with an MST of 46 days ($p > 0.05$) **B)** Mice were treated with 500 μg anti-CD8 or isotype control on days -3 and -4. ACAID was induced on day 0 with C57BL/6 splenic non-adherent cells. SC injections of C57BL/6 splenocytes were given on day +7. DTH challenge with mitomycin-C-treated C57BL/6 cells was on day +14. Negative control animals received an ear challenge only. *$p = 0.001$ between anti-CD8 and isotype control (N = 5). This experiment was repeated 3 times with similar results.

ACAID CD8$^+$ T cells and corneal immune privilege CD4$^+$CD25$^+$ Tregs act at the efferent arm of the immune response

Our original hypothesis posited that orthotopic corneal transplantation was tantamount to an AC injection of alloantigens and that the Tregs in mice with long-term surviving corneal allografts were, in fact, ACAID Tregs. Although our earlier experiment suggested that the CD8$^+$ Treg subset is either not induced or dispensable in corneal allograft acceptor mice, we nonetheless wanted to examine a possible therapeutic use of ACAID CD8 Tregs. Specifically, we decided to compare the efficacy of the ACAID CD8$^+$ Treg subset at suppressing alloantigen-sensitized immune cells (i.e., the efferent arm of the alloimmune response) to CD4$^+$CD25$^+$ Tregs supporting corneal immune privilege. Accordingly, CD4$^+$ effector T cells were isolated from the spleens of corneal allograft rejector mice and were mixed with either CD4$^+$CD25$^+$ Tregs from corneal allograft acceptor mice or CD8$^+$ Tregs from mice primed in the AC with C57BL/6 spleen cells (i.e., ACAID Tregs). BALB/c APCs pulsed with C57BL/6 alloantigens *in vitro* were added to each culture. The positive control consisted of CD4$^+$ T cells isolated from rejector mice, and the negative control consisted of naive CD4$^+$ effector cells cultured with naive CD4$^+$CD25$^+$ natural Tregs. Admixed cell cultures were injected into the ear pinnae of naive BALB/c mice in a LAT assay, and DTH was measured 24 hours later. The results demonstrate that CD4$^+$CD25$^+$ Tregs from corneal allograft acceptor mice and CD8$^+$ Tregs from ACAID mice suppressed DTH responses mediated by CD4$^+$ effector cells isolated from corneal allograft rejector mice (**Fig. 31**). Thus, the Tregs induced by AC injection of alloantigens (i.e., ACAID Tregs) and Tregs induced in corneal allograft acceptor mice represent different populations, yet both are capable of suppressing DTH responses by previously sensitized CD4$^+$ effector T cells.

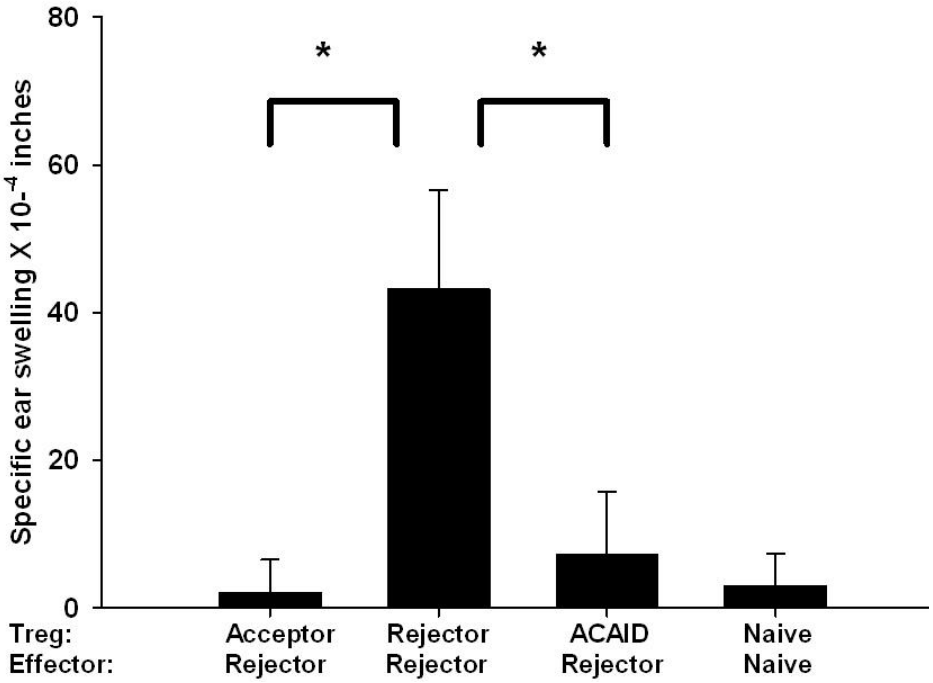

Figure 31. Efferent suppression by ACAID and corneal allograft-induced Tregs. $CD4^+CD25^+$ Tregs were isolated from acceptor BALB/c mice 3 weeks post-transplantation. ACAID $CD8^+$ Tregs were isolated on day 14 after AC priming with C57BL/6 non-adherent spleen cells. Effector $CD4^+$ T cells were isolated from corneal allograft rejector mice and mixed with the Tregs. DTH-positive control animals received rejector $CD4^+CD25^+$ Tregs and $CD4^+$ rejector effectors, and negative control animals received naive $CD4^+CD25^+$ Tregs and naive effectors. Prior to the LAT assay, individual cell innocula were supplemented with BALB/c APCs which were pulsed with C57BL/6 alloantigens *in vitro* for 24 hrs. *$p < 0.0001$ for acceptor versus rejector $CD4^+CD25^+$ Treg recipients; $p < 0.0005$ for ACAID $CD8^+$ Tregs versus rejector $CD4^+CD25^+$ Treg recipients ($N = 5$ per group). This experiment was performed twice with similar results.

CHAPTER FOUR

DISCUSSION

Clinical relevance of this study

Corneal transplantation is arguably one of the most successful forms of solid organ transplantation performed in humans. Early outcomes for corneal transplantation are typically excellent with up to 90% survival one year post surgery (97). However, retrospective inspection of corneal transplant registry data shows that the early observations stand in stark contrast to long term outcome. The probability of graft survival post surgery as estimated by Kaplan-Meier analysis plummets to 73% at 5 years, 62% at 10 years and 55% at 15 years (191). High-risk hosts with inflamed and vascularized graft beds experience even higher incidences of rejection with only 20% of patients bearing healthy grafts 15 years after initial keratoplasty (192). These long term studies demonstrate that although guaranteeing initial success, the immune-privileged status of the cornea does not ensure graft longevity. As the cornea's immune regulatory mechanisms wane and immunosuppressive regimens fail, immune-mediated graft rejection becomes the most important cause of graft failure (191). In the United States alone, 6000 to 8000 patients reject their transplanted corneas annually (www.nei.nih.gov/health/cornealdisease). With each corneal transplant costing approximately $20,000 and the incidence of allograft rejection increasing sharply with each regraft, there is a dire need for therapies that enhance corneal allograft survival (193).

In order to curtail immune-mediated graft failure, the work described in this dissertation investigated the mediators of experimental allograft rejection and tolerance. This study sheds

new light on classical paradigms in keratoplasty and reevaluates the role of the $CD4^+$ T cell lineages and the effect of their signature cytokines on the fate of experimental corneal allografts.

ROLE OF IL-17A IN CORNEAL ALLOGRAFT REJECTION

Th1 subset is not necessary for allograft rejection

In the classical paradigm of corneal allograft rejection, Th1-based immune responses are characterized by IFN-γ and IL-2 production and DTH responses directed to the donor's histocompatibility antigens that lead to graft rejection (29, 30). Accordingly, experiments were performed to determine whether elimination of IFN-γ and thus, classical Th1 responses, would promote survival of corneal allografts mismatched at the MHC and miH loci. Our results suggest that the Th1 arm of the immune response is not necessary for allograft rejection. Accordingly, the role of the newly identified $CD4^+$ T cell subset, the Th17 subset, was evaluated based on several reports linking the Th17 lineage to the pathogenesis of several autoimmune diseases and allograft rejection previously thought to be Th1-mediated (61-63).

Interestingly, our findings demonstrate that the Th17 cell subset is not necessary for allograft rejection. Instead, our experiments reveal a correlation between *in vivo* neutralization of IL-17A and loss of immune privilege. Moreover, IL-17A appears to be preferentially expressed by $CD4^+$ T cells from mice with surviving allografts. Our observations add support to the growing body of evidence highlighting the protective role of this cytokine. However, unlike the colitis and GVHD models where IL-17A appears to limit Th1-associated inflammation and differentiation (72, 73), our observations suggest that a completely distinct IL-17A-dependent regulatory mechanism is operating in corneal transplantation. It appears that the increased

incidence of rejection in IL-17A depleted hosts is not a result of the loss of Th1 cross-regulation or exaggerated DTH as previously believed.

Th2-mediated allograft rejection in IL-17A and IFN-γ deficient hosts

The classical paradigm of $CD4^+$ T cell cross-regulation posits that the Th2 lineage can cross-regulate Th1 responses, thereby suppressing the clonal expansion of Th1 cells (29). Thus, it has been proposed that tilting the alloimmune response toward a Th2 pathway would favor allograft survival. Initially, contradicting reports prevented a clear examination of the Th2 subset's role in allograft outcome. However, mounting evidence has lent credence to the notion that Th2-based immune responses instead exacerbate graft rejection instead of preventing it (42-44). The data presented here clearly demonstrate that the blockade of IFN-γ by anti-IFN-γ antibody treatment or the blockade of IL-17A by anti-IL-17A antibody treatment of graft recipients promotes the emergence of Th2 alloimmune responses, which in turn, exacerbate corneal allograft rejection. Additional functional evidence validating the role of the Th2 cell lineage as a mediator of allograft rejection was revealed in the adoptive transfer of the Th2 cells to T cell-deficient hosts, which led to a 100% incidence of rejection. Similarly, corneal allografts transplanted into anti-IL-17A-treated STAT6 KO mice, which do not generate IL-4-mediated functions including Th2 cell differentiation, do not experience the heightened graft rejection observed in IL-17A-depleted wild-type mice. The work described here is the first to show that the Th2 cells can independently mediate corneal allograft rejection.

However, our report is not the first to suggest that IL-17A mitigates Th2-based inflammation. Using a murine model of allergic asthma, Schnyder-Candrian *et al.* found that *in vivo* neutralization of IL-17 exacerbated allergic asthma, whereas administration of exogenous

IL-17 reduced pulmonary eosinophil recruitment and bronchial hyperreactivity (75). Th2 mediated eosinophilic-allograft rejection has also been described for cardiac and skin allograft rejection in mice (194-197). In contrast to those reports, the Th2-mediated graft rejection observed in IL-17A-depleted hosts appears to be independent of eosinophils. The lymphocytic infiltrate observed in the anti-IL-17A rejectors is reminiscent of the pattern observed in the unchallenged eyes of hosts with allergic conjunctivitis (44). In the aforementioned study, mice were challenged with pollen on one eye and the opposite eye was given a corneal transplant. The animals experienced a 100% incidence of rejection and had significant infiltration of T1/ST2^{+} Th2 T cells at the rejection site with no eosinophilic infiltrate. Further investigations into the mode of action of the Th2 cells will be required. Preliminary results suggest that the process might be dependent on the presence of both MHC and miH mismatches. Depletion of IFN-γ in mice mismatched at both MHC and miH loci leads to a significant exacerbation in allograft rejection. Interestingly, inhibition of IFN-γ in recipients with either the miH or MHC only mismatches instead promotes corneal allograft survival (171). To date, no report describing a Th2-based, eosinophil-independent mode of graft rejection is available and further investigation will be required for unraveling the mode of action of these cells.

Th17 is required for corneal immune privilege

The recently identified Th17 lineage has been designated as the central mediator of pathological tissue damage particularly in autoimmune diseases previously thought to be mediated by the Th1 subset (61-63). Although the paradigm is consistent in the field of autoimmunity, little evidence is available in the transplantation field for a functional role for Th17 cells in allograft rejection. Reports linking Th17 cells to allograft rejection are predominantly based on association rather than functional analysis. On the other hand, IL-17A

has previously been shown to have several regulatory and protective effects. In mouse models of colitis and GVHD, IL-17A has been shown to abate the severity of the disease by limiting Th1-associated inflammation (72, 73). Additionally, IL-17A has been shown to negatively regulate asthma via inhibition of DCs and chemokine synthesis in sensitized hosts (75). Finally, in an experimental model of uveitis, treatment with recombinant IL-17A causes a significant increase in the number of $CD4^+CD25^+$ Tregs and an abatement in the clinical signs of the disease (187). Our observations here add further support to the growing body of evidence highlighting the protective role of IL-17A and reveal a correlation between *in vivo* neutralization of IL-17A and loss of immune privilege. Moreover, IL-17A appears to be preferentially expressed by $CD4^+$ T cells from mice with surviving allografts. Similarly, FACS analysis suggests that the corneal allograft acceptor mice also have increased expression of splenic $CD4^+Ror\gamma T^+$ T cells compared to the rejectors (data not shown).

The findings that the Th17 T cell subset is not necessary for allograft rejection are in keeping with a recent report by Yamada and coworkers who reported that MHC-matched corneal allografts from the 129 mouse strain underwent rejection in 100% of C57BL/6 IL-17 KO or IFN-γ KO mice (198). However, because 100% of the 129 mouse strain corneal allografts underwent rejection in WT C57BL/6 mice, it was not possible to discern an effect of either IL-17 or IFN-γ on the immune privilege of corneal allografts. A recent report by Chen and coworkers also demonstrated that 100% of the C57BL/6 corneal allografts underwent rejection in IL-17 KO BALB/c mice (69). However, unlike our findings, Chen *et al*. noted that although 100% of the corneal allografts underwent rejection in IL-17-deficient hosts, the MST was slightly prolonged. It should, however, be noted that as with the Yamada *et al.* report , 100% rejection was observed in the untreated hosts in the Chen *et al.* model and hence, did not allow for an evaluation of the

role of IL-17A on corneal immune privilege. Nonetheless, the Chen *et al.* study suggests that IL-17A was produced during the early stage of corneal allograft rejection and that deletion of the IL-17A gene delayed, but did not prevent the rejection of corneal allografts. Thus, deletion of the IL-17A gene or *in vivo* treatment with anti-IL-17A fails to reduce the incidence of corneal allograft rejection.

The data presented here suggest that IL-17A prevents acute corneal allograft rejection by inhibiting the generation of a Th2-based immune response. However, in addition to Th2 cross-regulation, IL-17A might serve multiple functions. This inference is based on the observation that STAT6 KO hosts deficient in IL-17A still experience increased rejection although at a reduced tempo. These data suggest that maintenance of corneal immune privilege by IL-17A possibly involves additional IL-17A-dependent regulators. In summary, the current data also indicate that neither the Th1 nor the Th17 subset is required for allograft rejection and that inhibition of these lineages promotes the emergence of a Th2 subset that can independently mediate corneal allograft rejection.

ROLE OF IL-17A IN CORNEAL IMMUNE PRIVILEGE

IL-17A does not directly affect corneal cells

As previously mentioned, corneal immune privilege is a multifactorial mechanism based on factors intrinsic to the corneal tissue and AH (4). Accordingly, we assessed the local role of IL-17A on the ocular environment based on previous reports showing that the inflammatory cytokine IFN-γ promotes corneal immune privilege locally. IFN-γ has been shown to upregulate the expression of several molecules such as PD-L1, TRAIL, FasL, and IDO that stifle the efferent phase of the alloimmune response and protect the allograft (93, 157). However, in contrast to IFN-γ, IL-17A treatment of corneal cells *in vitro* did not significantly increase the expression of these immunoregulatory molecules.

In addition to upregulating the expression of suppressive molecules, an alternative mode of action for IL-17A might occur by endowing corneal cells with the ability to evade apoptosis. Based on reports that IL-17A in conjunction with TGF-β1, which is abundantly present in the AH, synergistically inhibits apoptosis in certain breast carcinoma cell lines (183), we tested whether the co-treatment with IL-17A and TGF-β1 could modulate the expression of pro- and anti-apoptotic factors, namely BCL-2, BCL-xL, and BAX to promote corneal cell survival. However, our results did not indicate a significant enhancement in the expression of the anti-apoptotic molecules, BCL-2 and BCL-xL, but did lead to a decrease in the expression of the pro-apoptotic molecule BAX. Additional experiments will be required to further validate a possible role for IL-17A in the evasion of apoptosis by corneal cells.

$CD4^+CD25^+$ Tregs in corneal immune privilege

Experiments from our group and others have demonstrated a critical requirement for $CD4^+CD25^+Foxp3^+$ Tregs in the maintenance of corneal immune privilege and allograft survival (77-79, 85). Antibody-mediated inhibition of the CD25 subset significantly exacerbates allograft rejection and adoptive transfer of $CD4^+CD25^+$ Tregs promotes allograft survival. Interestingly, and in contrast to other models, our investigations and those of others found no difference in the frequency of $CD4^+CD25^+Foxp3^+$ Tregs between corneal allograft acceptor and rejector mice. Instead, the ability of acceptor Tregs to promote graft survival is dependent on the functional status of the cells; that is, their levels of Foxp3 expression (85). Chauhan *et al*. demonstrated that the levels of Foxp3 was higher in the $CD4^+CD25^+$ population of acceptor mice when compared to the rejector mice (85). Using a CFSE-based *in vitro* suppression assay, we also demonstrated that $CD4^+CD25^+$ Tregs isolated from mice with clear corneal allografts 21 days post transplantation could significantly inhibit the activation of naïve $CD4^+$ effector cells compared to $CD4^+CD25^+$ Tregs from rejector mice. Further characterization revealed that the Treg population from acceptor mice could mediate suppression at the efferent phase of the immune response and were antigen-specific. These findings hold significant translational implications that will be discussed later.

Our investigations from *in vitro* transwell assays also indicate that acceptor $CD4^+CD25^+$ Tregs block the activation of $CD4^+$ T effector cells by a mechanism that is primarily contact-dependent. Tregs from acceptor mice express several contact-dependent suppressive molecules including GITR, CTLA-4, and membrane-tethered TGF-β1. The mechanisms that these membrane-bound molecules utilize to promote Treg suppression of $CD4^+$ T cells in corneal allograft rejection are currently being elucidated. It is known that interaction of membrane-bound

TGF-β1 with $CD4^+$ T cells induces anergy. The interaction between GITR, which is constitutively expressed by $CD4^+CD25^+$ Tregs, and GITR-L, which is displayed on APCs promotes the proliferation of $CD4^+CD25^+$ Tregs. Moreover, as mentioned previously, Hori *et al.* recently reported that GITR-L expression on corneal endothelial cells can stimulate expansion of Tregs which enhances corneal allograft acceptance (161). CTLA-4 expressed by $CD4^+CD25^+$ Tregs has been reported to enhance cell membrane expression of TGF-β1 by promoting the accumulation of TGF-β at the site of $CD4^+CD25^+$ Treg - $CD4^+$ T cell interaction (199). Interaction between CTLA-4 expressed by $CD4^+CD25^+$ Tregs and CD80/CD86 expressed by dendritic cells upregulates expression of indolamine 2,3-dioxygenase (IDO) (81). IDO catabolizes tryptophan, an amino acid that is crucial to T cell metabolism, leading to T cell starvation. Moreover, kynurenine which is a tryptophan metabolite produced by IDO enzymatic activity, promotes T cell apoptosis (200). While mouse models have shown that genetic enhancement of IDO expression results in prolonged corneal allograft survival in high-risk hosts, the direct or indirect role that IDO plays in $CD4^+CD25^+$ Tregs suppression remains to be determined (93).

The current study also provides interesting insight into Treg kinetics following corneal transplantation. First, $CD4^+CD25^+$ Tregs appear not to be necessary for the continued survival of corneal allografts beyond day 30, as administration of anti-CD25 and anti-IL-17A antibodies at this time point does not adversely affect long-term corneal allograft survival. Second, not only do hosts with long-term corneal allografts fail to display evidence of Tregs, but they are also unable to generate Tregs when a second corneal allograft is placed onto the contralateral eye. That is, secondary corneal allografts transplanted onto the contralateral eyes of hosts with primary corneal allografts that had been in place and clear for 90 days underwent rejection in 86% of the

hosts. Moreover, disabling the $CD4^+CD25^+$ Treg population in hosts bearing long-term clear primary allografts (i.e., >90 days) does not influence the survival of secondary corneal allografts in these hosts as 100% of the second corneal allografts undergo rejection. These results also suggest that long-term $CD4^+CD25^+$ Tregs and/or memory $CD4^+CD25^+$ Tregs are absent or non-functional beyond day 30.

$CD4^+CD25^+$ Tregs and the IL-17A connection

Several pieces of evidence hint at a possible link between IL-17A and Treg function. First, the tempo and incidence of rejection observed with inhibition of Tregs mirrored that of animals depleted of IL-17A. Additionally, the rejection observed with either anti-IL-17A or anti-CD25 treatment was Th2-mediated (data not shown). Finally, our own studies demonstrated that, compared to $CD4^+CD25^+$ Tregs from corneal allograft acceptor mice, $CD4^+CD25^+$ Tregs from anti-IL-17A treated rejector mice could not suppress naïve $CD4^+$ T cells *in vitro*. From these observations, we proposed that IL-17A is required for $CD4^+CD25^+$ Treg function.

Using a CFSE-based *in vitro* suppression assay, we confirmed that $CD4^+CD25^+$ Treg-mediated suppression required IL-17A. Previous studies using human peripheral blood have revealed the presence of IL-17-producing Tregs (172, 173). Accordingly, we next determined the source of the IL-17A. Anti-CD3ε stimulation and direct allostimulation of acceptor $CD4^+CD25^+$ Tregs yielded no IL-17A production. By contrast, stimulation of the splenic $CD4^+CD25^-$ fraction from corneal allograft acceptor mice produced significant levels of IL-17A. My results also show that exogenous IL-17A does not directly suppress $CD4^+$ T effector cells, but instead, potentiates the activity of naïve $CD4^+CD25^+$ Tregs. Further evidence establishing that IL-17A itself is not the suppressive factor that directly promotes corneal allograft survival comes from the *in vitro*

transwell assays, which indicate that acceptor $CD4^+CD25^+$ Tregs block the activation of $CD4^+$ T effector cells by a contact-dependent mechanism. Instead, results obtained using quantitative real-time PCR analysis on $CD4^+CD25^+$ Tregs used in the $CD4^+$ T cell suppression assays depleted of IL-17A revealed a downregulation of three surface bound molecules expressed by Tregs; GITR, CTLA-4, and membrane-bound TGF-β1. Additionally, simultaneous administration of blocking antibodies to all three molecules reduced suppression of $CD4^+$ T cells by $CD4^+CD25^+$ Tregs to levels seen with anti-IL-17A blockade. The current observations suggest that IL-17A is required by $CD4^+CD25^+$ Tregs to maintain or upregulate the expression of these various suppressive molecules (**Fig. 32**). Moreover, although our own studies and those of others (85) suggest a requirement for IL-10 for $CD4^+CD25^+$ Tregs mediated-suppression, IL-17A blockade does not appear to inhibit production of this soluble molecule (data not shown).

The observation that a proinflammatory cytokine such as IL-17A is needed for the generation and function of Tregs is not unprecedented. IFN-γ is necessary for the function of ACAID $CD8^+$ Tregs, and TNF-α and IFN-γ are needed for generating and potentiating $CD4^+CD25^+$ Tregs in other models of immune regulation (184-186, 190). Interestingly, when I further assessed expression of these inflammatory cytokines, I found significant levels of IFN-γ and TNF-α production by the same $CD4^+CD25^-$ T cells from corneal allograft acceptor mice. These results are exciting because they offer a new view into Treg biology suggesting that low level inflammatory responses can selectively activate Tregs. The data presented here would support this inference and would provide a sensible mechanism by which the Tregs could be activated to suppress efferently. Interestingly, it also appears that the cytokine profile of the limited inflammatory response, which is predominantly IL-17A in acceptor mice, might serve as an activation cue for Tregs. Additional work will indeed be required to test these hypotheses.

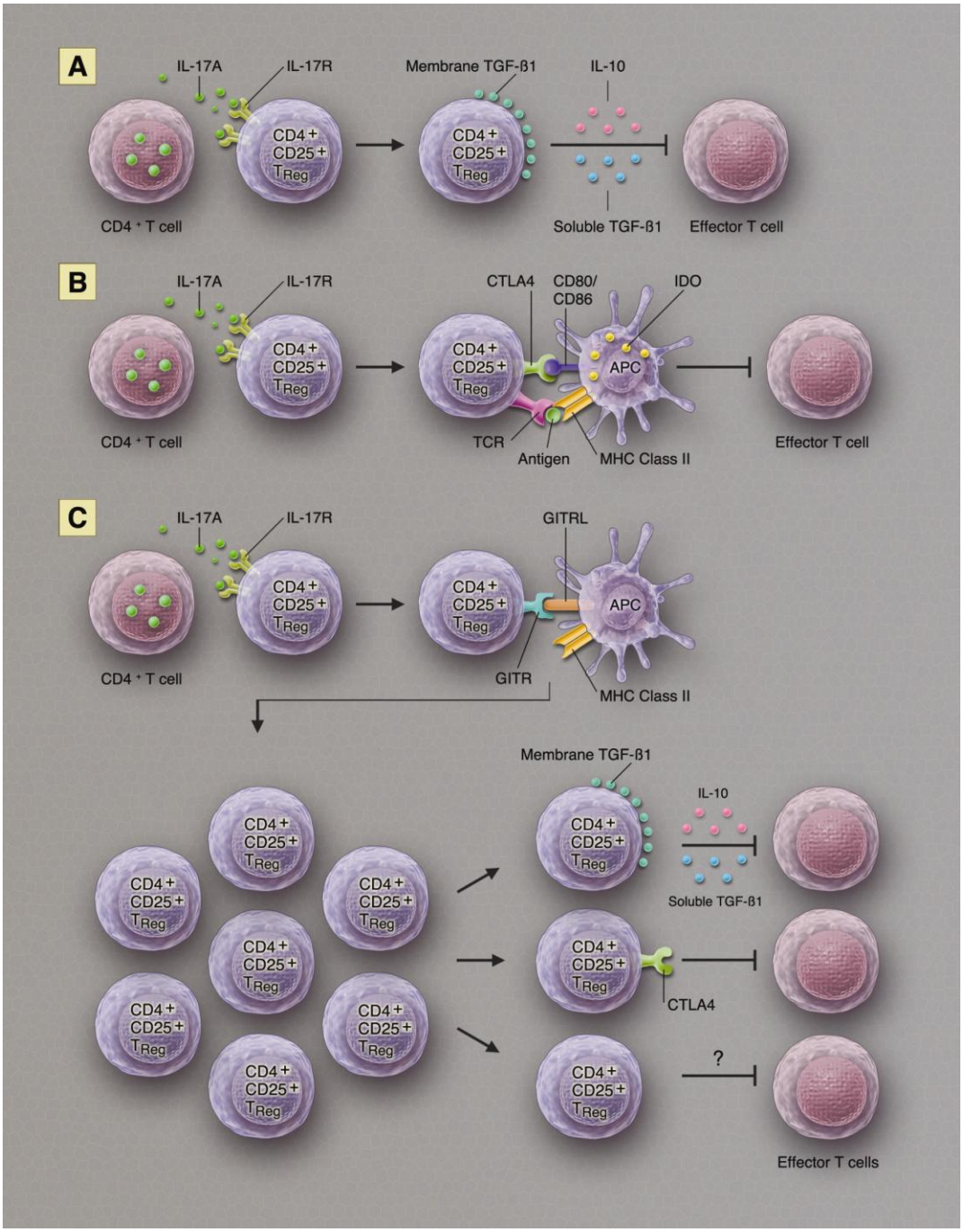

Figure 32. Potential pathways for IL-17-dependent T regulatory cell enhancement of corneal allograft survival. A) Direct immunosuppression by $CD4^+CD25^+$ Tregs entails production of IL-10 and TGF-β to directly inhibit effector T cell proliferation. **B)** Alternatively, $CD4^+CD25^+$ Tregs can regulate effector T cell responses via accessory cells using contact-dependent mechanisms. Interaction between CTLA-4 derived from $CD4^+CD25^+$ Tregs and CD80/86 present on dendritic cells upregulates the expression of indoleamine 2,3-dioxygenase which leads to effector T cell apoptosis. **C)** Interaction between GITR, expressed constitutively by $CD4^+CD25^+$ Tregs, and GITRL, present on APCs, can also expand the $CD4^+CD25^+$ Treg population to enhance regulation of alloimmune responses through multiple pathways.

ROLE OF ACAID IN CORNEAL IMMUNE-PRIVILEGE

Introduction of antigens into the AC elicits a unique form of systemic immune tolerance known as ACAID, which leads to antigen-specific suppression of DTH (99). Since orthotopic corneal allografts are placed directly over the AC of the eye, it has been proposed that the sloughing or shedding of corneal alloantigens into the AC would induce ACAID and promote allograft survival. Indeed, the antigen-specific suppression of DTH responses displayed by rodents with long-term clear corneal allografts is reminiscent of ACAID-mediated suppression of DTH (132). Additionally, inhibiting the induction of ACAID with procedures such as splenectomy or elimination of either NKT cells or γδ T cells hastens the tempo and increases the incidence of corneal allograft rejection (121, 134, 135). Induction of ACAID prior to keratoplasty by injection of donor alloantigenic cells into the AC significantly enhances corneal allograft survival in both the rat and mouse models of penetrating keratoplasty (34, 35). Based on these observations, we initially hypothesized that the IL-17A-dependent $CD4^+CD25^+$ Tregs that promoted corneal allograft survival and the ACAID Tregs were one in the same. However, the results indicate that two distinct forms of immune tolerance are involved in ACAID and corneal immune privilege respectively.

ACAID versus corneal immune privilege

Several studies had previously shown that ACAID requires the participation of two independent Treg populations. One population is $CD4^+$ and acts at the afferent arm to prevent induction of allosensitization, while the other Treg population is $CD8^+$ and acts at the efferent arm of the immune response to suppress DTH responses produced by previously sensitized T cells (99). Our initial investigation showed that inhibition of $CD4^+CD25^+$ subset with *in vivo*

treatment with anti-CD25 antibody prevented the induction of ACAID and robbed the corneal allograft of its immune privilege. Additionally, treatment with low dose cyclophosphamide, which has been shown to selectively inhibit $CD4^+CD25^+$ Treg function also inhibited the induction of ACAID and exacerbated allograft rejection, which is consistent with previous findings suggesting that the presence of $CD25^+$ Tregs correlates with corneal immune privilege. (176). Accordingly, based on the requirement for IL-17A by $CD4^+CD25^+$ Tregs in corneal immune privilege, we hypothesized that IL-17A would be required for ACAID. However, neutralization of IL-17A did not affect the development of ACAID, but, as stated earlier, had a profound effect on the survival of corneal allografts.

These observations establish an interesting paradigm where it appears that different $CD4^+CD25^+$ Treg subsets operate in ACAID and corneal allograft immune privilege. Several reports have recently hinted at this possibility. For instance, Saban *et al.* demonstrated that induction of ACAID causes a significant increase in the number $CD4^+CD25^+Foxp3^+$ Tregs while our results and those of others suggest that there is no significant increase in the frequency of $CD4^+CD25^+Foxp3^+$ Tregs in allograft acceptors (85, 201). Additionally and as mentioned previously, Hori *et al.* reported that GITR, in accordance with our own observations, is required for corneal allograft immune privilege but not for the induction of ACAID (161). These observations along with ours indicate the presence of two populations of $CD4^+CD25^+Foxp3^+$ Tregs. The Tregs that are responsible for corneal allograft immune privilege function at the efferent phase of the immune response, which is in contrast to ACAID $CD4^+CD25^+$Tregs which function afferently. Thus, it makes sense that IL-17A would only be required for promoting the immune privilege of corneal allografts. In that scenario, the IL-17A produced during the allosensitization phase would activate the Tregs that would then mediate suppression.

Furthermore, since IFN-γ had been shown to be required for the generation and activation of $CD4^+CD25^+Foxp3^+$ Tregs, I examined its role in both phenomena (184). As in previous experiments, depletion of IFN-γ exacerbated allograft rejection and also abolished ACAID. Studies by Cone and co-workers have shown that IFN-γ was required for the suppressive function of $CD8^+$ Tregs in ACAID (190). Although $CD8^+$ Tregs induced during ACAID did not need to produce IFN-γ, they could only exert their suppressive effects on DTH if they expressed the IFN-γ receptor and were capable of responding to IFN-γ. The results from Cone and coworkers therefore led us to assess whether $CD8^+$ Tregs were required for both corneal allograft immune privilege and ACAID. *In vivo* treatment with anti-CD8 antibody prevented the induction of ACAID, but the same anti-CD8 antibody treatment did not affect the immune privilege of orthotopic corneal allografts. The present findings, although in agreement with the work of Cone *et al.*, indicate that the IFN-γ requirement for corneal allograft immune privilege is independent of ACAID $CD8^+$ Tregs. Instead, it might be inferred that the $CD4^+CD25^+$ Tregs supporting corneal allograft survival also require IFN-γ. Interestingly, IFN-γ has been shown to be necessary for the generation of $CD4^+CD25^+Foxp3^+$ Tregs that mitigate EAE (184). In both human and murine systems, IFN-γ treatment leads to conversion of $CD4^+CD25^-$ T cells to $CD4^+CD25^+$ Tregs, an increased expression of Foxp3, and heightened suppressive activity (184). It remains to be determined if IFN-γ has a similar effect on the induction of $CD4^+CD25^+$ Tregs in recipients of corneal allografts, but the present results and previous findings by Chauhan *et al.*, which indicated that enhanced Foxp3 expression on $CD4^+CD25^+$ T cells correlated with corneal allograft survival, are consistent with this hypothesis.

Thus, the Tregs induced in ACAID are distinctly different from the Treg population that is induced by keratoplasty and supports the long-term survival of corneal allografts. Our present

results suggest the existence of two regulatory populations acting at the efferent phase of the immune response in what now appears to be two distinct phenomena. One population induced by the corneal allograft, is $CD4^{+}CD25^{+}$, and acts at the efferent arm of the immune response to suppress DTH responses by previously sensitized allospecific T cells. The second population is induced artificially by AC injection of alloantigens, is $CD8^{+}$, and also suppresses at the efferent phase of the immune response.

Requirement		Treatment	ACAID	Corneal Immune Privilege
Organ	Intact Spleen	Splenectomy	Abrogates	Abrogates
Cellular	CD25	Antibody mediated inhibition	Abrogates	Abrogates
	CD8	Antibody mediated depletion	Abrogates	No effect
	NKT	Antibody mediated inhibition Gene deletion	Abrogates	Abrogates
	γδ T cell	Antibody mediated inhibition	Abrogates	Abrogates
Cytokine	IL-10	Antibody mediated depletion	Abrogates	Abrogates
	TGF-β	Antibody mediated depletion	Abrogates	?
	IFN-γ	Antibody mediated depletion	Abrogates	Abrogates
	IL-4	Gene deletion	No effect	No Effect
	IL-17	Antibody mediated depletion	No effect	Abrogates
Surface molecule	PD-L1	Antibody mediated inhibition	No effect	Abrogates
	Fas-L	Antibody mediated inhibition Gene deletion	Abrogates	Abrogates
	IDO	Chemical inhibition	Abrogates	No effect
	TRAIL	?	?	?
	GITR	Antibody mediated inhibition	No Effect	Abrogates

Table 5. Differences between in cellular and molecular components of ACAID and corneal immune privilege. [202, 203]

THERAPEUTIC IMPLICATIONS

Current therapies for keratoplasty patients include topical application of corticosteroids, which by themselves carry the added risk of developing glaucoma and cataracts. Additionally, for high risk patients with a history of graft rejection, the use of systemic immunosuppressive agents only provides limited enhancement of allograft survival. Similarly, the immunosuppressive drugs have to be given for the lifespan of the patient and are accompanied by an increased incidence of infections and malignancy. A remedy to the current conundrum would involve developing treatment protocols that replicate regulatory mechanisms that lead to spontaneous development of transplant tolerance, thereby optimizing graft survival and limiting drug-induced side effects.

Protective role of IL-17A in corneal allograft survival

The current study provides interesting insight into possible modalities that might be developed in order to prevent allograft rejection and promote corneal immune privilege. First and foremost, the study shows a protective role for the cytokine IL-17A in keratoplasty. In contrast to most scientific reports which show an adverse association of IL-17A in other immune-mediated diseases (61-63, 65, 66, 68, 69, 204), the present study clearly demonstrates that this cytokine has a beneficial effect on the outcome of corneal transplantation. Thus, despite showing significant abatement of disease in patients suffering from psoriasis and rheumatoid arthritis, current experimental therapies directed at IL-17A inhibition would exacerbate instead of diminish corneal allograft rejection. Instead, topical administration of recombinant IL-17A in the clinic could be used as a therapeutic modality to promote allograft survival. In this scenario,

early administration of the cytokine would potentiate the Treg population locally, thereby inhibiting the generation of immune responses directed against the donor cornea.

Interestingly, it also appears that one of the primary functions of IL-17A is to cross-regulate the Th2 subset. This current finding provides additional opportunities for therapeutic intervention. For instance, our group has previously demonstrated that the presence of the Th2 cell subset in atopic hosts prior to keratoplasty significantly exacerbates allograft rejection. In short ragweed pollen induced allergic conjunctivitis and airway hyper-responsiveness, emergence of an allospecific Th2 immune responses led to the rejection of >90% of the corneal allografts (42-44). Based on several observations indicating that the absence of IL-17A promotes Th2-mediated pathology, it would be worth investigating whether topical or systemic administration of IL-17A could abate the incidence of rejection in atopic hosts.

Harnessing $CD4^+CD25^+$ Tregs to enhance corneal immune privilege

As mentioned earlier, $CD4^+CD25^+Foxp3^+$ Tregs constitute a key component of the regulatory machinery that induces and maintains tolerance to both self and alloantigens (19). The finding that $CD4^+CD25^+Foxp3^+$ Tregs are abundantly present in the grafts and in the peripheral blood of patients with tolerant liver, renal, and heart allografts suggests a positive association between the $CD4^+CD25^+Foxp3^+$ Tregs and enhanced transplantation outcomes (78, 79, 205).

In an attempt to develop Treg-based treatment modalities, the work here characterizes the mode of action of the $CD4^+CD25^+$ Tregs in promoting corneal allograft survival. Our current investigations suggest that the Tregs act at the efferent phase of the immune response, thereby providing a window of opportunity for therapeutic intervention through the adoptive transfer of Tregs in patients showing the initial clinical signs of corneal allograft rejection. The research

described here has also revealed that treatment of naïve $CD4^+CD25^+$ Tregs with recombinant IL-17A can significantly increase their suppressive potential to levels displayed by corneal allograft acceptor Tregs. Thus, instead of using Treg-based cellular therapy which carries the increased risk of *ex vivo* contamination and subsequent infections, systemic or topical administration of rIL-17A could be considered as a viable alternative. The present results with systemic administration of rIL-17A, however did not yield a significant change in the tempo of rejection, but did suggest a trend towards increased survival. However, early administration of higher doses of rIL-17A has been shown to abate the incidence of uveitis by increasing the number of $CD4^+CD25^+Foxp3^+$ Tregs (187). Hence, it might be expected that increased levels of rIL-17A might similarly lead to a significant enhancement in corneal allograft survival.

Several limitations regarding clinical application of the $CD4^+CD25^+$ Tregs have also been uncovered through our investigations. For instance, based on observations from our laboratory and those of others, the $CD4^+CD25^+$ Tregs which maintain corneal immune privilege are antigen-specific (85). Although the antigenic restrictions might hamper translational use of the $CD4^+CD25^+$ Tregs, several groups have reported encouraging progress regarding the use of natural Tregs in the clinical setting (19). Although the experimental cell therapies have not been applied to the field of transplantation, use of *ex vivo* expanded recipients $CD4^+CD25^+$ Tregs have been shown to significantly alleviate the symptoms of chronic GVHD (206). The adoptive transfers which were performed post-hematopoeitic stem cell transplantation allowed for a reduction in the immunosuppressive therapy, without significant adverse effects reported. Multiple clinical studies investigating the possible use of $CD4^+CD25^+$ Tregs to curtail GVHD are ongoing and have unanimously reported encouraging outcomes (19, 206).

Nonetheless, the Treg antigen specificity could be resolved based on two unique features of donor corneal tissue. First, donor corneas have a relatively long half-life *ex vivo*, and can be stored for up to one month before being transplanted. Second, the donor corneal tissues are also typically collected with the scleral rim which is discarded during the transplantation procedure. Taking advantage of those two features, one could imagine using the scleral rim as antigen to generate antigen-specific Tregs. During the time span when the cornea is still viable, Tregs could be generated *in vitro* using the patient's peripheral blood lymphocytes. APC's could also be isolated from the peripheral blood and pulsed with antigens prepared from the sclera rim from the corneal allograft donor specimen and co-cultured with the patient's peripheral blood lymphocytes in the presence of recombinant IL-17A. These Tregs would then be adoptively transferred to the host prior to transplantation. Additionally, frozen samples of scleral rim tissue could also be retrieved to provide antigen for future use in the generation of additional Tregs for adoptive transfers in the same patient. This personalized therapy would avoid the drawbacks stemming from the use of corticosteroids, and would hopefully provide long-term tolerance.

In addition to the antigen restrictions of the acceptor Tregs, we also focused our attention on the kinetics of the acceptor $CD4^+CD25^+$ Treg-mediated suppression, which is crucial to therapeutic implementation. The present findings indicate that the acceptor Tregs have a limited life-span and eventually dissipate. These results and additional observations from our laboratory indicate that multiple adoptive transfers of the $CD4^+CD25^+$ Tregs will be required in order to stably establish corneal allograft tolerance.

ACAID versus corneal immune privilege Tregs

The data presented here also differentiate between the regulatory T cell subsets that are induced by the cornea and AC. $CD4^+CD25^+Foxp3^+$ Tregs are essential to corneal allograft survival. By contrast, the $CD8^+$ Treg subset is required for ACAID, but not corneal immune privilege. Our results show that while the two subsets are unique to each phenomenon, they are both crucial for the suppression of alloimmune responses. The present observations provide critical insight that might lead to therapeutic use of both Treg subsets to achieve optimal tolerance. Indeed, it appears that the $CD4^+CD25^+Foxp3^+$ Tregs suppress more effectively, but do not lead to the deletion of alloreactive effector $CD4^+$ T cells. On the other hand, $CD8^+$ Tregs have been shown to induce apoptosis of the pathogenic $CD4^+$ T cells. Thus, combined use of both Treg subsets could reduce the number of cells and the number of adoptive transfers required to achieve graft tolerance.

Future studies

The body of knowledge unearthed through this dissertation will pave the way to new experimental pursuits, prime among them, the elucidation of the mechanisms used by Th2 cells to mediate graft rejection. Ongoing studies in our laboratory suggest that the process is abolished in recipient donor combinations where the mismatches involve only miH or MHC. For instance, compared to the C57BL/6 to BALB/c donor host combination, depletion of IFN-γ in either NZB to BALB/c (miH disparate) or BALB.B to BALB/c (MHC disparate) recipients does not exacerbate corneal allograft rejection despite the fact that these hosts develop Th2 cells. It appears that the Th2-mediated graft rejection is dependent on specific host and donor combinations with concomitant mismatches in miH and MHC required for Th2-mediated graft rejection. Additional experiments will be required to unravel the requirement for such allodisparities.

Moreover, while most Th2-based pathologies are eosinophil-mediated, the histological profile of the rejection observed with IL-17A depletion appears to be eosinophil-independent. We have previously reported a similar observation with atopic mice rejecting allografts placed in the contralateral eye that was not manifesting allergic inflammation *in situ*. A possible explanation for the absence of eosinophils in the rejected graft might be that the rejection process is antibody-mediated. For example, significant levels of circulating donor-specific antibodies have been detected in patients following keratoplasty (207-211). Unfortunately, mouse studies examining the role of alloantibody-mediated graft rejection have yielded mixed results (212, 213). Yet, the fact that Th2 cells are required for IgE class switching and that IgE can independently fix complement should spur investigations into the possible role of alloantibodies

into Th2-mediated corneal allograft rejection. Indirectly, these investigations might yield insight for curtailing corneal allograft rejections linked to atopy.

More importantly, the nature of the IL-17A-dependent cross-regulation of Th2 responses will need to be further examined. A report describing a requirement for IL-17A in the maturation of dendritic cells required for Th1 polarization would suggest that the cross-regulation is indirect (68). If future experiments uncover a direct inhibition of Th2 cells by IL-17A derived from the natural Tregs, these investigations might subsequently pave the way to the topical administration of IL-17A to prevent Th2-based abolition of corneal immune privilege or even ocular allergies.

As described earlier, a close connection exists between the outcome of keratoplasty and the presence of $CD4^+CD25^+Foxp3^+$ Tregs (78, 79, 205). Any additional work aimed at further understanding this field will initially need to scrutinize the site of action and kinetics of the Tregs. This may be achieved through time course experiments using transgenic mice expressing green fluorescent protein (GFP) tagged to the Foxp3 promoter. These studies will have to be designed such that the organ distribution of the regulatory T cells is assessed based on identical time points and clinical scores in order to control for the sporadic nature of allograft rejection.

My *in vitro* studies have also demonstrated that treatment of naïve Tregs with IL-17A significantly enhances their regulatory potential. It would be interesting to assess whether IL-17A treatment of $CD4^+CD25^+$ Tregs stably enhances this regulatory potential or whether it is transient. If the Tregs are endowed with increased suppressive abilities that are sustainable in the absence of the cytokine, the palliative role of IL-17A-pretreated Tregs could be evaluated through adoptive transfer studies. If the Tregs home to the cornea and act locally as suggested by

previous reports, one could consider topical administration of rIL-17A through eye drops or AC injections of the cytokine as a viable therapeutic pursuit.

With respect to $CD4^+CD25^+$ Tregs, several challenges to the translational use of Tregs were uncovered through these studies; the first one involved the antigen specificity of acceptor Treg-mediated suppression. Co-culture of natural Tregs with allogeneic APCs has been shown to generate antigen-specific Tregs in other settings (214). Donor cornea-derived APCs could be used to generate antigen-specific Tregs that promote graft survival. An additional constraint revealed in my studies is the transient nature of the Tregs. Experiments aimed at evaluating Tregs' half-life and the minimal number of Tregs required to support graft survival will be needed to circumvent this issue. Of the utmost importance will be the identification of stable markers for phenotyping Tregs**.** Also, the finding that $CD4^+CD25^+$ Tregs from corneal allograft acceptors express CTLA-4 and membrane TGF-β1 provides additional therapeutic incentives that might bypass the actual use of $CD4^+CD25^+$ Tregs. For instance, systemic administration of CTLA-4-Ig and subconjunctival administration of TGF-β1 liposomes to limit the incidence of corneal allograft rejection could be evaluated in mice.

My studies did not uncover any local effect of IL-17A. However, these studies encompassed a limited selection of anti-apoptotic molecules and apoptosis inducing molecules. With respect to the role of IL-17A in inhibiting corneal cell apoptosis, additional experiments looking into caspase 3 cleavage and phosphatidylserine flipping will be required to validate a possible role for IL-17A in the evasion of apoptosis by corneal cells. Recently, Hori *et al.* reported that GITRL expressed by corneal endothelial cells expands regulatory T cell populations locally to promote allograft survival (161). Additionally, although not described in mice, human corneal endothelial cells have been reported to express membrane-bound TGF-β1

(160). Based on our current results that IL-17A depletion leads to a decrease in the expression of GITR and membrane TGF-β1 on $CD4^+CD25^+$ Tregs, it would be interesting to assess if IL-17A is modulating the expression of these regulatory molecules on corneal cells such as GITRL, TGF-β, and CTLA-2α that potentiate Treg activity or directly induce effector T cell anergy (215).

Additional investigations will have to focus on the source of the IL-17A required for sustaining corneal immune privilege. The present study singled out $CD4^+CD25^-$ T cells as the primary producers of the IL-17A required for activating the $CD4^+CD25^+$ Tregs (216-218). Future studies will need to further define the cell subset responsible for Treg activation. A recent report suggests that myeloid derived suppressor cells (MDSCs) selectively activate $CD4^+CD25^+$ Tregs via IL-17A production (218). Indeed, the MDSCs sustaining the IL-17A dependent Tregs could be mimicking the $F4/80^+$ cell subset that is essential to ACAID and would provide a much larger population to work with compared to the less abundant and less easily purified $CD4^+CD25^+$ Tregs (4, 99).

In conclusion, the work described here investigated the role of IL-17A, and the immunoregulatory phenomenon, ACAID, in the maintenance of corneal immune privilege. The interaction of IL-17A with different $CD4^+$ T cell subsets and the corneal tissue was investigated and provided significant insight into its role in the immunobiology of keratoplasty. Finally, cellular and molecular deconstruction of ACAID and corneal immune privilege revealed that although sharing multiple components, the two phenomena are distinct and have mutually exclusive mediators. This study will hopefully increase the scientific community's understanding of the phenomenon and eventually pave the way to therapies aimed at improving corneal allograft tolerance and human health.

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