ROLE OF ALLERGIC CONJUNCTIVITIS IN INDUCING
CORNEAL TRANSPLANT REJECTION

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DEDICATION

To my parents,

Eulogio and Hilda Reyes

ROLE OF ALLERGIC CONJUNCTIVITIS IN INDUCING CORNEAL TRANSPLANT REJECTION

by

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Corneal allografts are the most commonly transplanted solid organs in humans and have a success rate of over 90%. This low incidence of graft rejection is largely due to the unique properties of the eye that decrease the likelihood of mounting an immune response, a phenomenon called “immune privilege”. Despite this characteristic, immune rejection remains the leading cause of corneal graft failure, indicating that immune privilege can be abolished. We used a model of corneal transplantation in which C57BL/6 corneal allografts transplanted to BALB/c mice experienced a 50% survival rate. This is in sharp contrast to the 100% rejection that occurs with other organ grafts exchanged between these two mouse strains. The 50%

survival rate decreased to 0% in mice with ongoing allergic conjunctivitis. We set out to delineate the mechanisms by which allergic conjunctivitis abolishes immune privilege and results in corneal allograft rejection.

Anterior chamber-associated immune deviation (ACAID) is initiated when foreign antigens, such as histocompatibility antigens sloughed from the cornea, enter the anterior chamber of the eye and induce a systemic down-regulation of immune responses. Studies have shown that abolishing ACAID increases the incidence of corneal allograft rejection. The hypothesis that allergic conjunctivitis abolished the induction of ACAID was tested and results showed that allergic conjunctivitis did not. Moreover, allergic conjunctivitis did not cause a qualitative or quantitative difference in cell-mediated immune responses to the donor's alloantigens.

Regulatory T cells (Tregs) have also been shown to be important in corneal allograft survival. Our results indicated that allergic conjunctivitis altered the Tregs that support corneal allograft survival. Th2 cytokines, namely IL-4, generated during allergic conjunctivitis, render effector cells resistant to the suppressive ability of Tregs. Furthermore, allergic conjunctivitis did not exacerbate corneal allograft rejection unless the host's effector T cells were able to respond to IL-4. Moreover, corneal immune privilege was restored in short ragweed (SRW) pollen-sensitized, allergic mice if they were isolated from SRW pollen for 14 days after receiving a corneal allograft. These results suggest that the exacerbation of corneal allografts in allergic hosts is due to the production of IL-4, which renders effector T cells resistant to Treg suppression.

TABLE OF CONTENTS

PREVIOUS PUBLICATIONS

1. Reyes, N.J., et al., *NKT cells are necessary for maximal expression of allergic conjunctivitis.* International Immunology, 2010. **22**(8): p. 627-36.

2. Cunnusamy, K., et al., *Two different regulatory T cell populations that promote corneal allograft survival.* Investigative Ophthalmology and Visual Science, 2010. **51**(12): p. 6566-74.

3. Reyes, N.J., et al., *Gammadelta T cells are required for maximal expression of allergic conjunctivitis.* Investigative Ophthalmology and Visual Science, 2011. **52**(5): p. 2211-6.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ACAID	Anterior chamber-associated immune deviation
APC	Antigen presenting cells
AC	Anterior chamber
SRW	Short ragweed
AH	Aqueous humor
NK	Natural killer
CNS	Central nervous system
CTLA-4	Cytotoxic T lymphocyte antigen-4
WT	Wild-type
B6	C57BL/6
AHR	Airway hyperreactivity
SAC	Seasonal allergic conjunctivitis
PAC	Perennial allergic conjunctivitis
VKC	Vernal keratoconjunctivitis
AKC	Atopic keratoconjunctivitis
BALF	Bronchoalveolar lavage fluid
α-GalCer	Alpha-galactosylceramide
NKT cells	Natural killer T cells
iNKT	Invariant NKT cells
i.v.	Intravenously
i.p.	Intraperitoneal
IC	Intracamerally

LIID	Lymphocyte-induced immune deviation
CTL	Cytotoxic T lymphocyte
MLR	Mixed lymphocyte reaction
DTH	Delayed-type hypersensitivity reaction
ELISA	Enzyme-linked immunosorbent assay
SC	Subcutaneous
HBSS	Hanks' balanced salt solution
MST	Median survival time
MRT	Median Rejection time
LAT	Local adoptive transfer
IDO	Indoleamine 2,3-dioxygenase
MHC	Major histocompatibility complex
GITR	Glucocorticoid-induced tumor necrosis factor receptor family-related gene
Foxp3	Forkhead box P3
Treg	Regulatory T cell
Th2	T helper 2
Th1	T helper 1
Th17	T helper 17
Ig	Immunoglobulin
LC	Langerhans cells
TGF-β	Transforming growth factor-beta
VIP	Vasoactive intestinal peptide
sFasL	soluble Fas ligand

CGRP	Calcitonin gene-related peptide
α-MSH	Alpha-melanocyte stimulating hormone
MIF	Macrophage migration inhibitory factor
CRP	Complement regulatory protein
SOM	Somatostatin
TRAIL	Tumor necrosis factor related-apoptosis inducing ligand
DC	Dendritic cells
PD-L1	Programmed cell death ligand-1
UVR	Ultraviolet radiation
HBO	Hyperbaric oxygen
EBAA	Eye Bank Association of America
CXCL1	Chemokine (C-X-C motif) ligand 1
CXCL9	Chemokine (C-X-C motif) ligand 9
CXCL10	Chemokine (C-X-C motif) ligand 10
MAPK	Mitogen-activated protein kinase
SCID	Severe Combined Immunodeficiency
VEGF	Vascular endothelial growth factor
VEGFR3	Vascular endothelial growth factor receptor 3
MFI	Mean fluorescence intensity
β2m	Beta2-microgobulin
CCTS	Collaborative Corneal Transplantation Studies
NK	Natural killer
H	Histocompatibility

iTregs	induced Tregs
gld	generalized lymphoproliferative disorder

CHAPTER ONE

INTRODUCTION

Anatomy of the Cornea

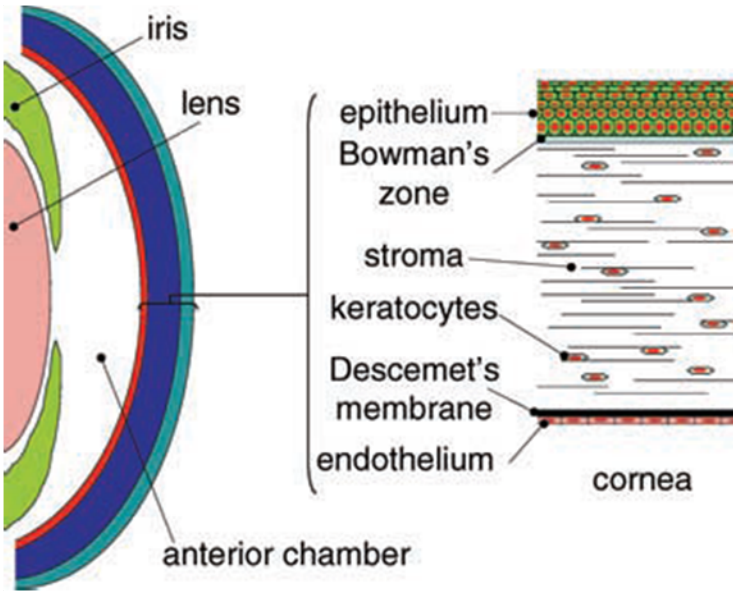
The cornea is an avascular and transparent tissue that acts as the primary barrier of the eye and provides two-thirds of the eyes optical power []. The cornea consists of three cellular layers: epithelium, stroma, and endothelium, and two interfaces: the Bowman and Descemet membranes (Figure 1) [1].

The epithelial layer is 4 to 6 layers thick and covered with a mucinous tear film produced by conjunctival goblet cells and lacrimal glands [1]. The epithelium makes up 10% of the total thickness of the cornea and functions as the first barrier to the outside environment. Three cell types populate the epithelium. The first two to three layers consist of squamous superficial cells that are joined by tight junctional complexes important for preventing pathogens from entering the deepest corneal layers. This layer is followed by wing cells that posses similar tight junctions. The deepest layer is adjacent to the Bowman's layer and is made up of basal cells. These cells are the only cells in the epithelium capable of proliferating and gradually migrate to the surface of the cornea to become wing cells and subsequently superficial cells [1]. Corneal epithelial cells have an average lifespan of seven to ten days due to continued division, differentiation, and migration of epithelial stem cells from the limbus, the junction between the cornea and the conjunctiva [2, 3]. Under normal conditions, limbal epithelial stem cells are also responsible for the regeneration of the epithelium after injury. Maintenance of the corneal epithelium is essential for protection of the stroma and the endothelium.

The corneal stroma is a collagenous layer that makes up about 85% of the thickness of the cornea. It differs from other collagen structures due to its transparency that is a result of the organization of collagen fibers and extracellular matrix [1]. Keratocytes in the stroma synthesize

collagen and glycosaminoglycans, which are responsible for absorbing and retaining large amounts of water, giving it its solid but elastic form [1]. Regulation of this hydration is important for maintaining transparency and is the function of the Descemet's membrane and the corneal endothelium. Conditions such as keratoconus where there is thinning of the stroma, causes the cornea to change to a conical shape, impairing the refractive index of the cornea [3]. This condition leads to very high myopia and astigmatism.

The corneal endothelium is comprised of a single layer of cells and is crucial for maintaining a state of deturgescence, or relative hydration that is necessary for corneal transparency [4]. Unlike corneal epithelial cells, endothelial cells in humans do not proliferate [5]. The most important physiological function of the endothelium is to regulate the water content in the stroma that is necessary for transparency. Endothelial cells contain Na^+, K^+ ATPase pump sites that promote the outflow of water from the stroma to the anterior chamber, thereby maintaining corneal clarity [3, 6]. Without proper stromal hydration, the stroma becomes saturated, compromising the structure of the cornea [3]. While all three layers of the cornea are susceptible to immune-mediated rejection, the corneal endothelium is the most important target when corneal allografts undergo rejection. An increase in hydration due to corneal disorders, inflammation, or physical disruptions of the endothelial layer results in deterioration of pump function, stromal swelling, and loss of transparency and vision [3, 6].



George and Larkin. Am J Transplant, 2004. 4(5): p. 678-85.

Figure 1. Anatomy of the cornea. The cornea consists of three layers. The corneal epithelium functions as a barrier protecting the cornea and preventing potential pathogens from entering. The stroma functions mainly for absorbing and retaining large amounts of water. The corneal endothelial layer is responsible for water transport between the stroma and aqueous humor (AH).

History of Corneal Transplantation

Corneal transplantation has been performed on human subjects for more than 100 years. In 1776, Erasmus Darwin suggested that opaque corneal tissue could be replaced, which would restore vision in the blind [7]. In 1813, Karl Himly also suggested that opaque corneas from animals could be replaced with transparent corneas from animals. This idea was not realized until 1837 when Samuel Bigger, an Irish surgeon, who was in captivity in Africa, performed the first successful penetrating allograft on a pet gazelle that had corneal scarring [7]. The success of Bigger sparked hope that corneal transplantation was achievable. In 1838, Richard Sharp Kissam attempted to transplant a pig cornea onto a human eye without the use of anesthesia. Kissam transplanted a portion of a pig's cornea onto the eye of a man that was blind due to an opaque cornea using 4 interrupted sutures [8]. There was only short-term success as the cornea became opaque within two weeks and was atrophied within a month [7]. It was not until 1905 that the first successful transplant was performed by Austrian ophthalmologist, Edward Zirm. Today, over 40,000 corneal transplants are performed annually in the US [9].

There are several different types of corneal transplantations. Lamellar keratoplasty involves selective replacement of diseased layers of the cornea while leaving healthy layers in place. This type of keratoplasty requires that the Descemet's membrane and endothelium are left in place since they provide the scaffold onto which the donor tissue can be laid. In endothelial keratoplasty, only the patient's endothelium and Descemet's membrane are replaced. Penetrating keratoplasty involves a complete removal of all the corneal tissues. Regardless of the type of corneal transplant, the goal is always to restore corneal function and, ultimately, visual clarity. According to the Eye Bank Association of America (EBAA), in 2009, 21% of penetrating keratoplasties were performed for patients with keratoconus, a condition in which the cornea

slowly changes from the normal round shape to a conical shape. Other indications for corneal transplants are summarized in Table 1 [9].

Indications for Penetrating Keratoplasty	
Post-cataract surgery edema	3,622
Keratoconus	5,092
Fuchs' Dystrophy	1,387
Repeat corneal transplant	4,085
Other degenerations or dystrophies	2,779
Microbial changes	637
Mechanical or chemical trauma	919
Congenital opacities	498
Post-refractive surgery	116
Other causes of corneal opacification or distortion	4,358
Total Indications for Penetrating Keratoplasty	23,493

Table 1. Total Number of Penetrating Keratoplasties Performed Annually in the US. In 2009, a total of 42,606 tissues were used within the U.S. Out of those, 23,269 were used in penetrating keratoplasties (PK) for various corneal diseases and disorders. About 55% of corneal transplants performed are for patients between the ages of 51-70 [9].

Ocular Immune Privilege

Corneal transplantation is the most common and successful form of solid organ transplantation in humans [10]. Several groups have shown the five-year survival rate of corneal transplants ranges between 88-90% [11-13], while others have found the rate to be approximately 75% [14]. Interestingly, these rates do not differ from the 5-year survival rates of 69% and 74% for kidney and heart transplants, respectively [15]. There are several striking differences between corneal transplants and those from other organs such as the kidney or heart. For one, kidney and heart transplants require the use of systemic immunosuppressive treatment, while corneal transplants rely only on topical corticosteroid treatment [16]. In addition, unlike kidney and heart transplants where HLA typing is critical for their survival, HLA typing is not routinely used for corneal transplants [17]. Moreover, a study published by the Collaborative Corneal Transplantation Studies (CCTS) found that HLA matching did not have a significant effect on corneal graft survival. Rather, results suggested that ABO blood group incompatibility had a bigger impact on the rate of rejection [17].

In rodent studies, most donor-host combinations result in more than 50% acceptance of corneal grafts mismatched at the entire major histocompatibility complex (MHC) and minor histocompatibility (H) gene loci [18, 19]. By contrast, similar donor-host combinations in skin allografts results in 100% rejection [20]. In combinations in which the donors and recipients differ at only the MHC class I or II loci, rejection occurs in less than 35% and 10%, respectively [10]. These observations demonstrate the unique immune privilege of the eye.

The concept of the eye as a site of immune privilege was first seen in the 1837 by van Dooremaal, a Dutch ophthalmologist, who observed prolonged survival of a mouse skin graft placed in the anterior chamber (AC) of the dog eye [21]. It took an additional 75 years until Peter

Medawar recognized extended survival of foreign skin grafts in the AC of rabbits that was not seen when placed elsewhere in the body and coined the term "immune privilege" [22]. Medawar established the role of the immune system in transplantation and concluded that the absence of conventional lymphatic draining of the eye sequestered antigens and therefore isolated the grafted tissue from immunological attack, a theory that became known as "immunological ignorance" [21]. Evidence for this theory was seen in studies in which skin allograft rejection between hosts with an existing corneal allograft and hosts without a corneal allograft was the same; therefore, it was concluded that orthotopic corneal allografts placed in avascular corneas failed to sensitize the host to donor alloantigens [23]. Furthermore, corneal allografts placed into vascularized corneal graft beds experienced increased rejection in humans, rabbits, and mice [10, 24]. What was not evident was that the growth of blood vessels also induced lymph vessel formation and increased the number of antigen presenting cells in the corneal graft bed [25]. The theory of immunological ignorance came under scrutiny with the observation that corneal allografts did in fact elicit an alloimmune response [26]. Early reports indicated systemic emigration of alloantigens from the ocular environment [23, 27]. Kaplan and Streilein further challenged this theory by showing that allogenic (F1) lymphoid cells injected intracamerally (IC) led to the production of antibodies against histocompatibility antigens [28]. Interestingly, while humoral immunity was generated, cell-mediated responses were dampened since rejection in orthotopic donor-specific skin allografts in the IC-primed hosts was delayed [28-30].

There are other organs and tissues in the body such as the pregnant uterus, brain, hair follicles, and the testes where immune privilege exists [31]. These sites share multiple anatomical, physiological, and immunoregulatory factors that prevent the induction of both innate and adaptive immune responses. This immune compromise protects these sites from

immune-mediated inflammation that might impair function of these vital tissues and threaten the survival of the host. In the eye, the cornea's avascularity and absence of lymphatics limits antigen sampling at the graft/host interface. In addition, unlike the skin that has a dense network of a subset of resident antigen presenting cells (APC) that are potent activators of the immune response called Langerhans cells (LC), the placement and phenotype of LC in the cornea differs significantly. For one, the corneal bed is devoid of MHC class II^+ LC. Trauma to the central cornea causes a rapid migration of MHC class II^+ LC and significantly compromises graft survival [32, 33]. Restricting the access to alloantigens, either by the absence of lymphatic drainage or by having the corneal bed devoid of mature LC, impairs or prevents the sensitization phase of the alloimmune response.

While MHC class Ia is expressed on all nucleated cells, expression in the eye and brain is absent or weak [34-36]. This reduced expression protects cells from these sites from lysis by $CD8^+$ cytotoxic T lymphocytes (CTL) in the event of a viral infection. Protection from CTL-mediated killing is important for these sites since the cells are incapable of regeneration. The absence of MHC class Ia does make these sites vulnerable to killing by natural killer (NK) cells, which are programmed to kill MHC class I negative cells [37]. To circumvent this, cells in the eye and the fetus express nonclassical MHC class Ib molecules, HLA-G and HLA-E, which interact with NK inhibitory receptors [38, 39].

The aqueous humor (AH) that fills the AC offers another layer of protection because it contains an array of soluble molecules that prevent the activation of immune cells and inhibit the production of inflammatory mediators (Table 2). Delayed-type hypersensitivity (DTH) reactions result in necrosis and damage to bystander cells [40]. The AH contains four factors that inhibit DTH: TGF-β, α-melanocyte-stimulating hormone (α-MSH), vasoactive intestinal peptide (VIP),

and calcitonin gene-related protein (CGRP) [41-45]. The AH, like the pregnant uterus, contains indoleamine dioxygenase (IDO). This enzyme catabolizes tryptophan, an amino acid that T cells cannot make on their own, and is crucial for T cell survival [46].

In addition to the suppressive molecules in the AH, the eye is protected by the various cell membrane-bound molecules expressed by the endothelial cell layer (Table 3). During corneal allograft rejection, allospecific T cells migrate into the cornea through the iris and limbal blood vessels. Molecules such as FasL, TRAIL, and PD-L1 are expressed on corneal cells and induce apoptosis of infiltrating leukocytes [47-50]. Murine studies in which BALB/c mice received corneal transplant from C57BL/6 (B6)-gld/gld (generalized lymphoproliferative disorder) mice, which lack functional FasL expression, experienced a significantly higher rate of graft rejection [51]. FasL is also expressed on fetal cytotrophoblasts and protects the allogenic mouse fetus from killing by activated T cells [52]. The central nervous system (CNS) also expresses FasL on neurons, astrocytes, oligodendrocytes, microglia, and the vascular endothelium [53]. Like FasL, TRAIL is a member of the tumor necrosis factor family of membrane proteins that induces the apoptosis of inflammatory cells [40]. PD-L1 expression in the eye has also been shown to contribute to its immune privilege. Studies in which BALB/c mice received a corneal allograft from B6 mice that lack PD-L1 experience an increased incidence of graft rejection [54, 55].

Lastly, alloantigens that enter the pregnant uterus, brain, or eye induce the generation of regulatory T cells (Tregs) that produce a dynamic down-regulation of systemic immune responses. In the eye, this phenomenon is known as anterior chamber-associated immune deviation (ACAID) and will be discussed in detail in the next section. Collectively, immune privilege is believed to protect tissues that cannot regenerate from immune-mediated injury.

Molecule	Effect on Immune Response
TGF-β	Suppresses the activation of T cells, NK cells, and macrophages; promotes APC to induce tolerance
VIP	Inhibits T cell activation and differentiation; inhibits DTH; suppresses secretion of TNF-α and IL-17
sFasL	Suppresses neutrophil recruitment and activation
IDO	Catalyzes tryptophan degradation inhibiting T cell proliferation
CGRP	Inhibits production of nitric oxide by macrophages
α-MSH	Inhibits T cell secretion of proinflammatory cytokines; suppresses neutrophil effector function
MIF	Inhibits NK cell killing
CRP	Disables complement activation
SOM	Suppresses IFN-γ production by activated T cells; induces production of α-MSH

Table 2. Immunosuppressive Molecules Present in the Aqueous Humor. TGF-β, transforming growth factor-beta; VIP, vasoactive intestinal peptide; sFasL, soluble Fas ligand; IDO, indoleamine 2,3-dioxygenase; CGRP, calcitonin gene-related peptide; α-MSH; alpha-melanocyte stimulating hormone; MIF, macrophage migration inhibitory factor; CRP, complement regulatory proteins; SOM, somatostatin [31, 56].

Molecule	Effect on Immune Response
mTGF-β	Suppresses the activation of T cells, NK cells, and macrophages
GITRL	Promotes proliferation of regulatory T cells
TRAIL	Induces apoptosis of inflammatory cells
FasL	Promotes apoptosis of T cells
PD-L1	Induces apoptosis of T cells

Table 3. Immunosuppressive Molecules Expressed by the Cornea. TGF-β, transforming growth factor-beta; GITRL, glucocorticoid-induced tumor necrosis factor receptor family-related gene ligand; TRAIL, tumor necrosis factor related-apoptosis inducing ligand; FasL, Fas ligand; PD-L1, programmed cell death ligand-1 [31].

Anterior Chamber-Associated Immune Deviation (ACAID)

As previously mentioned, one of the earliest observations of ocular immune privilege was made in the 1940s by Medawar who noted extended survival of skin allografts placed in the AC of rabbit eyes. Medawar concluded that immune privilege was a product of sequestration of foreign antigen and cells in the AC due to the absence of lymphatic drainage [22]. It took 25 years before this theory was disproven by Kaplan and Streilein when they discovered that IC-injected semi-allogenic lymphoid cells not only activated humoral immunity, but the ensuing immune response was deviated to a state of tolerance [29]. This phenomenon was initially termed lymphocyte-induced immune deviation (LIID); however, subsequent studies done by Niederkorn and Streilein highlighted the critical role of the AC compartment [28-30, 40]. Further investigation led to an extensive understanding of this phenomenon called ACAID.

ACAID is characterized by antigen-specific suppression of T helper 1 (Th1) cell-mediated immune responses such as DTH and the production of non-complement-fixing antibodies [21, 57, 58]. ACAID is induced when antigens such as viral proteins, soluble proteins, or histocompatability antigens are injected into the AC or by corneal transplantation [28, 59, 60]. In some models, CTL responses are also suppressed, while in others, CTL responses are intact [61, 62]. In addition, the humoral response elicited is that of non-complement fixing antibodies [58, 63].

The last thirty years have led to an extensive understanding of the mechanism of ACAID. Studies have shown that within forty-eight hours after injection of the antigen, the antigen is captured by $F4/80^{+}$ APC which travel from the iris/ciliary body into the AC. Because of the TGF-β-rich environment of the eye, these APC preferentially secrete IL-10, and are therefore tolerance-inducing APC. Within 48 hours, APC leave the eye and migrate to the thymus where

they induce the generation of $CD4^{-}CD8^{-}NK1.1^{+}$ thymocytes. These thymocytes, along with $F4/80^{+}$ APC, migrate to the spleen where interactions occur with B cells, NKT cells, and γδ T cells [60, 64, 65]. While these interactions are not well understood, they lead to the generation of $CD4^{+}$ T afferent suppressor cells and $CD8^{+}$ T efferent suppressor cells [21, 66].

There is indirect evidence of the importance of ACAID in corneal transplantation. For example, corneal transplants placed directly over the AC of the eye facilitate the shedding of alloantigens into the AC which can lead to the induction of ACAID. In murine models of corneal transplantation, recipients with clear grafts displayed antigen-specific suppression of DTH responses that was similar to the suppression found in ACAID [31, 67, 68]. Furthermore, inducing ACAID by injection of donor antigen into the AC of corneal allograft recipients prior to transplantation resulted in enhancement of corneal allograft survival [69, 70]. Blocking the induction of ACAID by splenectomy or deletion of natural killer T (NKT) and γδ T cells led to an increased incidence and a swifter tempo of corneal allograft rejection [59, 71-73].

It is important to note that certain conditions can cause the eye to lose its immune privilege and, subsequently, increase the risk of graft rejection. These factors include loose sutures or early suture removal [74], corneal vascularization [74, 75], an increasing number of regrafts [74], bilateral grafts [76, 77], ocular inflammation, uncontrolled glaucoma [78], atopy, and dry eye states [79-81].

Conditions that Abolish Immune Privilege

Lymph and Blood Vessel Formation

The absence of blood, and more importantly, lymph vessels, is a unique property of the cornea [82-84]. In animal models, induction of hemangiogenesis prior to corneal transplantation by placing sutures into the cornea prior to surgery significantly increases the incidence and

tempo of rejection [24, 25]. Ksander and colleagues found that these high-risk hosts readily induce donor-specific CTL that are not present in normal, avascular corneas [24]. Moreover, adoptive transfer of $CD8^+$ T cells isolated from mice with a pre-vascualized graft bed into severe combined immunodeficiency (SCID) mice that subsequently received a corneal allograft resulted in 100% rejection [85]. This suggests that under high-risk conditions, CTL that do not normally develop in low risk conditions are responsible for the increased incidence of corneal allograft rejection.

What was not evident early on was that blood vessel formation in the cornea coincided with lymph vessel formation. Blood vessel formation was believed to facilitate the egress of APC to the draining lymph nodes (LN) as well as migration of effector cells back to the graft site; however, there is growing evidence suggesting that lymphogenesis, and not hemangiogenesis, is responsible for this trafficking [25]. Vascular endothelial growth factor (VEGF)-C binds to VEGF receptor 3 (VEGFR3) to induce lymphogenesis [84]. Albuquerque and colleagues have shown that corneal epithelial cells secrete VEGFR2, which blocks VEGF-C and subsequently inhibits lymphogenesis [82]. Moreover, administration of soluble VEGFR2 inhibited lymphangiogenesis, but not hemangiogenesis that was induced by corneal sutures or by transplantation and enhanced corneal allograft survival [82]. Another group found that administration of α5β1 integrin, which selectively blocks lymphogenesis while preserving hemangiogenesis, reduced corneal allograft rejection [86].

Further evidence that lymphogenesis aids in the trafficking of APC to the draining LN as well as enhancing the migration of effector T cells back to the corneal allograft was seen in studies done by Amescua et al. in which high-risk vascularized recipients produced chemokine (C-X-C motif) ligand 1 (CXCL1) which is not found in normal avascular corneas [87]. This

chemokine is responsible for stimulating T cells to secrete chemokine (C-X-C motif) ligand 9 (CXCL9) and chemokine (C-X-C motif) ligand 10 (CXCL10) involved in the recruitment of T cells to the graft site [88-90]. Treating high-risk vascularized hosts with anti-CXCL1 antibody resulted in restoration of normal avascular corneal beds, while administration of CXCL1 converted normal hosts to high-risk hosts and thus increased graft rejection [87].

Langerhans cells

Increased migration of APC to the regional LN after corneal transplantation dramatically increases graft rejection. LC are bone-marrow derived APC that are potent activators of T cells [91]. Studies in rodent models demonstrate that under normal conditions the corneal epithelium is devoid of MHC class II^+ LC [32]. However, it has been shown that during infection or even following surgery there is rapid migration of LC from the limbus to the center of the corneal epithelium [32]. Moreover, in this study, infiltrating donor-derived LC increased the immunogenicity of the corneal allograft since grafts with donor-specific LC underwent 96% corneal allograft rejection compared to 55% rejection that occured in LC-free corneal buttons [19, 92]. In the murine model of corneal transplantation, the use of ultraviolet radiation (UVR) and hyperbaric oxygen (HBO) depleted LC and promoted corneal allograft survival [93]. Conversely, LC^+ grafts increased rejection up to 80% from 40% seen in LC^- grafts, similar to what is seen in the rat model. These results are in agreement with Ray-Keil et al. who also found that HBO and UVR treatment dramatically enhanced corneal allograft survival [94].

Allergic diseases

One type of allergic response occurs when the body produces an immediate hypersensitivity reaction in response to an allergen. Allergic diseases, such as allergic conjunctivitis and allergic AHR, also elevate the risk for corneal graft rejection [80, 95-98].

Studies have found that when recipient mice have an ongoing allergic response, either allergic conjunctivitis or allergic airway hyperreactivity (AHR), they experienced significantly increased incidences and swifter tempos of rejection compared to non-allergic hosts [80, 98, 99]. Further detail on how allergic diseases exacerbate corneal allograft rejection will be discussed in the section titled Corneal Allograft Rejection on page 24.

The development of allergic reactions begins with the sensitization phase during which IgE antibody is produced. While the exact mechanism by which B cells isotype switch to IgE is not clear, it is evident that Th2 cytokines, notably IL-4 and IL-13, play an important role. Once exposure to the allergen has occurred through repeated contact and IgE antibody has been produced, an individual is considered sensitized. Following sensitization, type I allergic responses may consist of two reaction phases: an early phase and a late phase. The early phase reaction occurs within minutes of allergen exposure and is initiated when the allergen crosslinks specific IgE antibodies bound via FcεRI receptors on the surface of ocular mast cells resulting in the release of histamine, leukotrienes, proteases, prostaglandins, and cytokines [100, 101]. The late phase occurs six to twelve hours later and involves the infiltration of inflammatory cells, especially eosinophils into the reaction area. Many allergens are airborne particles such as dust or pollen and affect mucosal tissues of the nose, airways, and eyes. Other allergens include foods, insects, and medications such as aspirin or penicillin. Allergic reactions that are caused by foods may affect the gastrointestinal tract and can lead to abdominal pain, bloating, and vomiting [102, 103]. Food allergies also can trigger potentially fatal anaphylaxis and food allergies are associated with atopic dermatitis [102, 103]. An allergic reaction most frequently seen in young children is atopic dermatitis, an eczema that is characterized by pruritus [104]. Allergic rhinitis, also known as hay fever, affects between 10 and 20% of the population in the US and Europe

and is characterized by watery nasal secretions, itching, and nasal congestion [105]. Allergic asthma is a chronic obstructive disease of the lower airways. It affects ~5% of the population of the Western world and has increased in prevalence over the past two decades [106]. Characteristics of allergic asthma are airway inflammation, mucus production, enhanced eosinophilia, elevated IgE, and induced AHR in response to a variety of stimuli [107, 108].

Mucosal allergies affect the eye and encompass a variety of ocular diseases that are typically confined to the conjunctiva [109]. These ocular diseases include: seasonal allergic conjunctivitis (SAC), perennial allergic conjunctivitis (PAC), vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC). SAC constitutes 90% of the allergic eye disease cases and is most often caused by grass, tree, and pollens. PAC comprises 5% of cases and occurs year round due to exposure to dust mites, animal dander, and/or outdoor molds [110-112]. SAC and PAC are more mild forms of conjunctivitis, while AKC and VKC represent more severe forms. AKC is often associated with eczema, and dermatitis and can cause severe damage to the ocular surface, leading to corneal scarring and vision loss [113]. VKC affects less than 1% of allergic eye disease cases. It recurs seasonally in the spring with symptoms that include pruritis that is induced by nonspecific stimuli that include wind, dust, bright light, hot weather, or physical exertion [112]. In addition to increased number of eosinophils, basophils, and mast cells in conjunctival biopsies, VKC its most remarkable characteristic is “giant” papillae [112]. In allergic conjunctivitis, the early phase reaction is characterized by tearing, lid edema, conjunctival edema (chemosis), and vasodilatation of conjunctival blood vessels. The late phase reaction involves the infiltration of eosinophils, into the conjunctiva [100].

T helper (Th2) cells are critical for the development of type I allergies [114]. Naïve helper T cells differentiate into four major subsets: Th1, Th2, T helper 17 (Th17), and Tregs.

What determines the development of each subset is the cytokine milieu present at the time of differentiation. For Th2 cells, IL-4 is the key cytokine important in their differentiation. Upon IL-4 binding to the IL-4 receptor, receptor-associated Jak 1/3 are activated and induce the phosphorylation of the tyrosine residues on the cytoplasmic tail of the IL-4 receptor [114]. These phosphorylated tyrosines become docking sites for STAT6 transcription factor, which upon docking becomes phosphorylated. Subsequently, STAT6 translocates into the nucleus and turns on the expression of GATA3 [115]. GATA3 remodels the IL-4 locus into a more accessible condition and leads to the production of signature Th2 cytokines, IL-4, IL-5, and IL-13 [116].

The functions of IL-4 include the differentiation of Th2 cells, B cell activation, immunoglobulin (Ig) isotype switching to IgE, and the inhibition of Th1 differentiation [117]. The Th2 cytokine IL-5 activates and promotes the maturation of infiltrating eosinophils [118]. Another Th2 cytokine, IL-13, is implicated in increasing mucus production in allergic responses [119]. Although Th2 cells are important in ocular allergies, they alone may not be sufficient to produce an allergic response. Other cells such as NKT and γδ T cells are thought to contribute to Th2-mediated immune responses [120].

NKT cells

NKT cells are a unique subgroup of lymphocytes that differ from conventional αβ T cells in that they produce both Th1 (IFN-γ) and Th2 (IL-4) cytokines upon stimulation [121, 122]. NKT cells recognize lipid antigens presented by a nonclassical MHC molecule, CD1d [123]. Two types of NKT cells have been described. Type I NKT cells are defined by their invariant T cell receptor (TCR) Vα14Jα18 in mice and Vα24Jα18 in humans and are referred to as invariant NKT (iNKT) cells [123]. Type II NKT cells express a diverse non-Vα14 TCR [124]. Studies on NKT cells have produced contradictory results as to their function. While some studies implicate

NKT cells in controlling immune responses against infection and tumors, others suggest that they act to protect self-tissue from damage caused by pro-inflammatory immune responses [123]. NKT cells have been implicated in allergen-induced, immune-mediated diseases such as allergic asthma and contact dermatitis [119, 120, 125]. Recent studies in mice have found that NKT cells play an essential role in the development of AHR, a hallmark of asthma. Mice lacking type I NKT cells have a decreased ability to develop AHR, display reduced airway eosinophilia, show decreased levels of IL-4 and IL-5 in the bronchoalveolar lavage fluid (BALF), and have reduced antigen-specific IgE compared to WT mice [106, 120, 126, 127]. These results are in agreement with those found by Akbari et al. who found that AHR does not develop in $J\alpha18^{-/-}$ mice, which lack type I NKT cells, or $CD1d^{-/-}$ mice, which lack both type I and type II NKT cells [119]. These findings demonstrate that NKT cells are required for maximal pulmonary eosinophilic infiltration, Th2 cytokine production, and elevated serum IgE levels in mice with AHR. The role of NKT cells in allergic asthma in humans is controversial. While some studies demonstrate a pronounced increase in the numbers of NKT cells in the BALF of patients with allergic asthma [128-130], other studies have not seen this increase [131-133].

γδ T cells

γδ T cells preferentially express TCR V regions in distinct tissues [134]. γδ T cells can produce Th2 cytokines and they may also contribute to the onset of allergic reactions. In asthmatic patients, Th2 cytokines produced by γδ T cells were increased compared to healthy controls [135]. Studies in mouse models have shown that using γδ T cell-deficient mice prevented the mice from developing AHR [136]. Additionally, Zuany-Amorin et al. showed that γδ T cell-deficient mice exhibited low specific IgE and IL-5 release and a decrease in T cell infiltration compared to WT mice [137]. This response was restored when IL-4 was

administered, suggesting that γδ T cells were necessary for secretion of IL-4, Th2-mediated inflammation, and for allergic airway inflammation. In contrast, other groups have shown that $CD8^+$ γδ T cells inhibit late airway response and have no significant role in modulating airway inflammation [138]. These conflicting results can be explained by findings demonstrating that there are two subsets of γδ T cells: $V\gamma1^+$, which enhances AHR and $V\gamma4^+$, which suppresses AHR [139, 140].

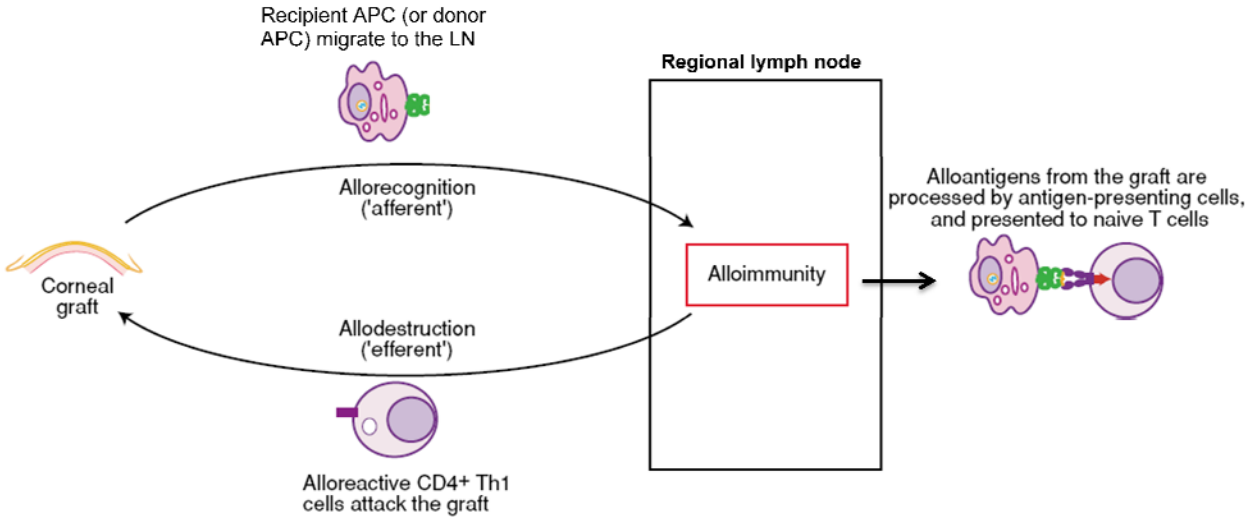
Unlike other forms of high-risk hosts, few studies have analyzed how allergic diseases exacerbate corneal allograft rejection.

Corneal Allograft Rejection

Immune rejection remains the leading cause of corneal transplant failure. All three layers of the cornea can be the target of immune rejection. The epithelial layer has a rapid turnover, and therefore, destruction of this layer is not as significant. In contrast, endothelial cells cannot replicate, and therefore, any damage to this layer is irreversible. Currently, the primary strategy for preventing acute inflammation is topical glucocorticosteroid treatment [141].

Much of what is known about immune rejection of corneal allografts has come from animal models. In immune rejection, it was originally believed that recipient recognition of donor MHC alloantigen played a major role in allograft rejection. However, subsequent studies have shown that the array of multiple minor histocompatibility (H) antigens is a greater barrier to graft survival than MHC antigens [142]. There are two distinct recognition pathways for provoking alloimmune responses: the indirect and direct pathways. In the direct pathway, recipient T cells recognize intact donor MHC molecules on the surface of donor APC [6]. In the indirect pathway, donor histocompatibility molecules, either MHC or minor H, are taken up and processed by host APC and presented in the context of self MHC molecules to host T cells [6,

143, 144]. In the direct pathway, donor MHC molecules are presented to host T cells by donor APC without processing. In both the direct and indirect pathways APC home to a regional LN where they initiate the alloimmune response. This process is known as the afferent arm of the immune response [145]. The efferent arm is that component of the adaptive immune response that leads to the destruction of the allograft. Alloreactive T cells leave the lymph nodes and migrate to the corneal tissue where they initiate the destruction of the graft [146].



Qian and Dana. Expert Rev Mol Med, 2001. 3(18): p. 1-21.

Figure 2. Afferent and efferent arm of the alloimmune response. Alloantigens are introduced into the eye by the corneal allograft. In the afferent arm of the alloimmune response, these alloantigens are captured and processed by recipient APC that migrate to the regional LN (or donor APC migrate directly to the regional LN). These APC present antigen and prime effector T cells, which undergo clonal expansion. In the efferent arm of the alloimmune response, antigen-specific T cells migrate back to the graft site where they mediate graft rejection.

$CD4^+$ T cells in Corneal Allograft Rejection

Immune rejection is the leading cause of corneal allograft failure. Studies have shown that corneal allograft rejection is mediated by $CD4^+$ T cells as depletion of $CD4^+$ T cells by antibody treatment or gene deletion resulted in increased survival [147-149]. There are several subsets of $CD4^+$ T cells that include Th1, Th2, Th17, and Tregs.

Th1 cells

Th1 cells are characterized by their expression of transcription factor T-bet and their secretion of IFN-γ. There is evidence suggesting that Th1 cells are the main mediators of corneal allograft rejection. In both rodent models of corneal transplantation and human patients that have undergone keratoplasty, there is an upregulation of TNF-α and IFN-γ mRNA during allograft rejection [150, 151]. It has been shown that deletion of TNF-α receptor I or TNF-α receptor 2 results in a reduction in corneal allograft rejection in the mouse model [152]. Moreover, corneal allograft rejection is strongly correlated with the DTH response to donor antigen and the production of IFN-γ [153, 154]. The exact mechanism by which Th1 cells mediate rejection is not known; however, there are several candidate molecules that may act as mediators. The adhesion molecules VCAM-1 and ICAM-1 on endothelial cells are both upregulated by TNF-α and can potentially mediate corneal allograft rejection by recruiting mononuclear cells [155, 156]. Administration of antibodies directed against ICAM-1, its integrin LFA-1, and the VCAM-1 integrin VLA-4 reduced corneal allograft rejection [157, 158]. While some studies have found that prolonged graft survival is associated with decreased levels of IFN-γ and IL-2 [159], others have shown that deletion of IFN-γ by antibody treatment or gene deletion resulted in significant exacerbation of corneal allograft rejection [160]. This suggests that IFN-γ may be necessary for

establishing immune privilege. It is important to note that in these IFN-γ deficient mice there is an upregulation of Th2 cytokines and Th2 alloimmune effector cells [160].

Th2 cells

Th2 cells are characterized by the expression of the transcription factor GATA-3 and the production of IL-4, IL-5, and IL-13. In the classic paradigm of immunology, IFN-γ cross-regulates IL-4 [161]. It was therefore proposed that deviating the immune response to a Th2 response prior to receiving a corneal allograft there would increase survival [80]. Beauregard and colleagues employed a mouse model of allergic conjunctivitis using short ragweed (SRW) pollen to induce a Th2 response in mice prior to receiving a corneal allograft. Recipient mice with allergic conjunctivitis had an increased incidence and swifter tempo of rejection [80]. Histological analysis showed that allergic recipients had the presence of T1/ST2$^+$ Th2 cells that were not seen in normal controls [80]. Further dissection of the rejection occurring in an allergic setting demonstrated that Th1 cells are still needed to mediate full rejection, as co-adoptive transfer of Tim3$^+$CD4$^+$ Th1 cells and T1-ST2$^+$CD4$^+$ Th2 cells into SCID mice was the only combination that mirrored the tempo and incidence of rejection seen in WT allergic hosts [162].

The increased incidence of corneal allograft rejection in allergic mice was hypothesized to be caused by antigen-non-specific local inflammation [80]. Results demonstrated that a corneal allograft placed on the opposite eyes that had not been exposed to SRW pollen experienced 95% graft rejection compared to the normal 50%. Furthermore, mice with allergic AHR that received corneal allografts also experienced an increased incidence in corneal allograft rejection [98]. These studies suggest that corneal allograft rejection is due to a systemic alloimmune effect rather than a local inflammatory response. These data are in line with Flynn et al. who found that mice that are both sensitized and challenged with SRW pollen have decreased

corneal allograft survival compared to normal mice and mice that have only been sensitized, but not challenged [99]. Interestingly, Flynn and colleagues showed that topical treatment with antihistamines alone did not prevent the increased corneal allograft rejection caused by allergic conjunctivitis, which is further evidence that the exacerbation of corneal allograft rejection was the result of a systemic, not local, effect of allergic conjunctivitis.

T helper 17 (Th17) cells

In addition to Th1 and Th2 subsets of $CD4^+$ T cells, more recently, $CD4^+$ Th17 cells have been identified. Th17 cells are characterized by the expression of the transcription factor RORγT and by the secretion of IL-17A, IL-17F, IL-21, and IL-22 [163, 164]. Th17 cells have been implicated in a variety of autoimmune diseases that were previously thought to be mediated by Th1 cells [165-167]. In corneal transplantation, one study found that gene deletion of IL-17 did not result in a prolongation or enhancement of corneal allograft survival, suggesting that Th17 cells were not involved as effector cells in graft rejection [168]. In contrast, depletion of IL-17A resulted in an increased incidence of corneal allograft rejection from 50% to 90% [160]. Further characterization in IL-17 depleted hosts revealed that the $CD4^+$ T cell subset had a Th2 cytokine profile and when adoptively transferred could mediate corneal allograft rejection [160]. Further dissection of the role of IL-17 in corneal allograft survival showed that IL-17A is necessary to generate contact-dependent suppression by $CD4^+CD25^+$ Tregs [169].

Tregs

$CD4^+CD25^+$ Tregs are characterized by the expression of transcription factor Foxp3 and make up 5-10% of the $CD4^+$ T cell population. Tregs are categorized as being either natural or induced [170]. Natural Tregs (nTregs) have a T cell receptor (TCR) that is specific for autoantigens and thus blocks autoimmune responses. By contrast, induced Tregs (iTregs) are

generated in the periphery from $CD4^+CD25^-$ T cells and are implicated in alloantigen tolerance [170]. In corneal transplantation, Tregs have been shown to play an important role in allograft survival. Treatment of BALB/c recipient mice with anti-CD25 antibody resulted in increased corneal allograft rejection [171]. It has also been shown that Tregs from mice that have accepted their corneal allografts (graft acceptors) suppressed the proliferation and activation of naïve effector T cells [169, 172]. Soluble suppressive molecules such as IL-10 and active transforming growth factor-β1 (TGF-β1), which are known to be secreted by Tregs, are necessary for the induction of ACAID and corneal allograft immune privilege [173]. In addition, membrane-associated suppressive molecules such as membrane-bound TGF-β1, glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR), and cytotoxic T-lymphocyte antigen-4 (CTLA-4) have been shown to be necessary for Treg-mediated suppression since blocking of all three molecules leads to inhibition of suppression of T cell activation [174].

GITR is highly expressed on $CD4^+CD25^+$ Tregs; however, the exact mechanism of how GITR on Tregs mediates suppression is not well understood. GITR, which is expressed on both Tregs and $CD4^+CD25^-$ effector T cells, interacts with GITR ligand (GITRL) expressed on dendritic cells (DC), B cells, and macrophages [173]. It has been suggested that during the initiation of an immune response, GITR on T cells interacts with GITRL on APC and promotes T cell survival [175]. As the response progresses, the Treg population, also expressing GITR, will interact with APC, and in the presence of IL-2 produced by effector T cells, will undergo expansion [175]. These Tregs are then able to exert their suppressive function on effector T cells. CTLA-4 is another molecule that also mediates its effect through Treg-APC interactions. CTLA-4 on Tregs interacts with CD80/CD86 on DC resulting in the expression of IDO by DC [176].

Current Investigations

Patients undergoing keratoplasty who have an avascular corneal graft bed have an acceptance rate of 90% [177]. Certain conditions such as corneal vascularization [74, 75], multiple regrafts [74], bilateral grafts [76, 77], ocular inflammation, uncontrolled glaucoma [78], dry eye states, and allergic diseases [79-81] cause the eye to lose its immune privilege, thus decreasing the success rate for a corneal transplant. The study presented here will focus on one such condition: allergic conjunctivitis.

Allergic conjunctivitis is an immediate hypersensitivity reaction mediated by a Th2-immune response. The first goal was to determine if NKT and γδ T cells were needed for the maximum development of SRW pollen-induced allergic conjunctivitis. Once this was determined, the focus of the study aimed to examine the mechanism of how allergic conjunctivitis abolishes immune privilege and exacerbates corneal allograft rejection in a mouse model of corneal transplantation.

Studies on corneal transplantation have shown the importance of ACAID in corneal allograft survival. The first aim of this study focused on determining if allergic conjunctivitis abolished the induction and expression of ACAID. The exact mechanism by which a corneal allograft is rejected is not well characterized; however, it is known that rejection is dependent on $CD4^+$ T cells. The next aim focused on determining if allergic conjunctivitis causes a quantitative difference in the $CD4^+$ T cell response compared to non-allergic hosts. We also examined if allergic conjunctivitis caused a qualitative difference in the alloimmune response by generating donor-specific CTL.

More recently, $CD4^+CD25^+$ Tregs have been shown to play a major role in corneal allograft survival. The effect of allergic inflammation, and more specifically of Th2 cytokines,

on Tregs that support corneal allograft survival has not been studied. In other models of tolerance such as long-term surviving heart allografts or pancreatic islet cell allografts, IL-4 is frequently detected and is associated with tolerance [178-180]. By contrast, in a sheep model of corneal transplantation, corneas expressing IL-4 induced eosinophilia, inflammation, and rejection [181]. The final aim of this study was to determine if allergic conjunctivitis alters the Tregs that support corneal allograft survival.

Overall, the focus of this study was to dissect how allergic diseases, more specifically allergic conjunctivitis, alter the systemic immune response that leads to increased corneal allograft rejection.

CHAPTER TWO

MATERIALS AND METHODS

Animals

B6 (H-2^b) and BALB/c (H-2^d) mice were purchased from the UT Southwestern Mouse Breeding Facility. Jα18$^{-/-}$BALB/c mice were generated as previously described and kindly provided by Dr. Masaru Taniguchi, RIKEN Research Center for Allergy and Immunology, Yokohama, Japan [182]. CD1d$^{-/-}$BALB/c mice were kindly provided by Dr. Mark A. Exley, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA. TCR-δ$^{-/-}$ mice were purchased from The Jackson Laboratories. Jα18$^{-/-}$, CD1d$^{-/-}$, and TCR-δ$^{-/-}$ mice were bred at the University of Texas Southwestern Medical Center Animal Resource Center. For grafting experiments, eight- to ten-week old female wild-type (WT) BALB/c, IL-4Rα$^{-/-}$ BALB/c, and B6 mice were purchased from Taconic Farms (Germantown, NY). The animal studies were approved by the Institutional Review Board of the University of Texas Southwestern Medical Center. Animals were housed and cared for in accordance with the Association for Research in Vision and Ophthalmology statement about the Use of Animals in Ophthalmic and Vision Research.

Induction of allergic conjunctivitis by active immunization

Allergic conjunctivitis was induced as previously described [183]. Briefly, mice were immunized with 50 μg of SRW pollen (International Biologicals, Piedmont, OK, USA) in 5 mg of alum (Thermo Fisher Scientific Pierce, Rockford, IL, USA) by intraperitoneal (i.p.) injection on day 0. Allergic conjunctivitis was induced by a multihit topical challenge in which immunized mice were given 1.5 mg of short ragweed pollen in 10 μl PBS in the right eye from days 10 to 16. Mice were examined clinically for signs of immediate hypersensitivity responses 20 minutes after each topical challenge with SRW pollen or PBS. For grafted mice, SRW pollen was applied

three times a week after corneal allograft. For assessing allergic conjunctivitis in IL-4Rα$^{-/-}$ mice, mice were examined clinically for signs of immediate hypersensitivity responses 20 minutes after each topical challenge with SRW pollen or PBS. Each parameter (lid edema, tearing, conjunctival vasodilatation, and conjunctival edema) was scored on a scale ranging from 0 to 3 [183]. A score of 0 indicated that there was no evidence of the respective parameter; 1+ = mild response distinctly greater than the naïve control; 2+ = moderate change in respective parameter that could be noted by biomicroscopy, but not with the naked eye; and 3+ = severe response that could be perceived with the naked eye. For transplantation experiments, mice were challenged on day 17 with a B6 corneal allograft.

Reconstitution of NKT cells

Spleen cells were processed as described above. T cells were isolated using a magnetic microbead system (Miltenyi Biotec, Auburn, CA, USA). Splenocytes were incubated with biotin-antibody cocktail for 10 minutes followed by an anti-biotin microbead incubation for 15 minutes at 4 °C. Unattached beads were washed off with bead buffer. Cells not attached to magnetic bead were collected as the enriched T cell population. T cell negative cells were retained in the column and eluted with bead buffer. Enriched T cells were stained with PE mouse anti-mouse NK1.1 and APC hamster anti-mouse TCR-β and incubated for 30 minutes. The stained cells were isolated using the FACS Aria. NK1.1^{+} TCR-β^{+} cells were used as purified NKT cells. NKT cells (2.0×10^5 - 4.5×10^5 cells/recipient) were i.v. injected into naïve B6 mice prior to immunization with SRW pollen. NKT negative spleen cells were used as a control. Ten days later mice were challenged topically with SRW pollen as previously described.

***In vivo* treatment of anti-CD1d, anti-IL-4, and GL3**

Mice were treated with intravenous (i.v.) injections of rat anti-mouse CD1d mAb (hybridoma HB323; American Type Culture Collection, Manasas, VA, USA) and rat-IgG (Sigma-Aldrich, St. Louis, MO, USA) isotype control 3 times a week (50 μg/injection) beginning 7 days prior to immunization. Mice were treated with intraperitoneal (i.p.) injections of 1mg rat anti-mouse IL-4 mAb (hybridoma HB188; American Type Culture Collection) or rat-IgG isotype control (Sigma-Aldrich) beginning the day they received a corneal transplant and 3X/week thereafter. GL3 antibody was produced from hybridoma cells and was provided by Dr. Leo Lefrancois (University of Connecticut, Farmington, CT). Mice were depleted of γδ T cells with i.p. injections of hamaster anti-mouse GL3 mAb (400 μg/injection) given two times a week beginning 5 days before immunization with SRW pollen.

Induction of allergic conjunctivitis by adoptive transfer of primed $CD4^+$ T cells

Spleens were removed from $J\alpha18^{-/-}$ or WT BALB/c mice 17 days after sensitization with SRW pollen. $CD4^+$ T cells were isolated using a magnetic microbead system (Miltenyi Biotec). Single-cell suspensions of splenocytes were prepared by gently processing between the ends of two sterile frosted slides. Splenocytes were incubated with anti-mouse CD4-coated magnetic beads in bead buffer (0.5% BSA in PBS, pH 7.2) for 15 minutes at 4 °C. Unattached beads were washed off with bead buffer. Cells not attached to magnetic beads passed through the column and were discarded. $CD4^+$ cells attached to magnetic beads were retained in the column, and eluted with bead buffer. One spleen equivalent of enriched $CD4^+$ T cell suspensions ($7.0 \times 10^6 – 9.0 \times 10^6$ cells/recipient) were injected i.v. into naïve BALB/c (or B6) mice (< 2% were double positive for NK1.1 and TCR-β). Four days later mice were challenged topically with SRW pollen as previously described.

Cytokine Enzyme-Linked Immunosorbent assay (ELISA)

Mice were euthanized 17 days post challenge and their spleens removed. Single-cell suspensions of splenocytes were prepared by gently processing between the ends of two sterile frosted slides. 1 x 10^7 cells/ml were incubated with 25 μg/ml of soluble short ragweed pollen extract (Greer Labs, Lenoir, NC,USA) for 48 h in 2 ml of RPMI supplemented with 10% FCS, 2 mM L-glutamine (Cambrex, Charles City, IA, USA), 1 mM sodium pyruvate (Cambrex), 1% penicillin-streptomycin-Fungizone (Cambrex), 1% nonessential amino acids (Cambrex), 1% HEPES buffer (Cambrex), and 5 x 10^{-5} M 2-mercaptoethanol (2-ME) (Sigma-Aldrich). Six hours before harvest, 1 μg/ml ionomycin (Sigma-Aldrich) and 25 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) were added to stimulate cytokine release. ELISAs for IL-4, IL-5, IL-13, and IFN-γ were performed on culture supernatants according to the manufacturer's instructions (R&D Systems).

Histology

Eyes from mice were removed day 17 post challenge and fixed in 10% formalin for histology. Paraffin-embedded tissue sections were stained with Congo Red. Differential cell counts were performed counting all inflammatory cells in the forniceal conjunctiva in the histologic section of each mouse. Inflammatory cells were counted in masked fashion by two investigators and were recorded as either eosinophils, neutrophils, or mononuclear.

AC Injection of Alloantigenic Cells

Mice were anesthetized as described above. A glass micropipette (approximately 80 μm diameter) was fitted onto a sterile infant feeding tube (no. 5 French, Professional Medical Products, Greenwood, SC) and mounted onto a 0.1 ml syringe (Hamilton Co., Whittier, CA). An

automatic dispensing apparatus (Hamilton Co.) was used to inject plastic nonadherent B6 spleen cells (1×10^5 cells in 4 μL) into the AC of BALB/c mice.

Delayed-type hypersensitivity (DTH) Assay

DTH was measured using a conventional ear swelling assay. An eliciting dose of $1x10^6$ B6 cells were injected subcutaneously (SC). Seven days later, 1×10^6 mitomycin C–treated (400 μg/ml) B6 spleen cells in 20 μL of Hanks' balanced salt solution (HBSS) was inoculated into the right ear. The left ear served as a negative control and was injected with 20 μL of HBSS without cells. Results were expressed as alloantigen-specific ear swelling response = (24 h measurement – 0 h measurement) for experimental ear – (24 h measurement – 0 h measurement) for negative control ear.

Orthotopic corneal transplantation

Allergic or naïve WT BALB/c and IL-4Rα$^{-/-}$ mice were given orthotopic corneal allografts onto the right eye from naïve B6 mice as previously described [147]. For allergic mice, grafts were placed 17 days after the initial SRW pollen immunization. For pre-vascularised mice, sutures were placed in three different locations on the cornea for two weeks and removed the day the mice received a corneal transplant. Grafts were scored based on opacity, edema, and neovascularization twice per week. Degree of opacity ranged between 0 to 4: 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+.

Preparations of APC

Single-cell suspensions of spleen cells were prepared from naïve BALB/c mice. BALB/c APCs were isolated by incubating the splenocyte suspension in two 100-mm Primaria plates (5 ml/plate) per spleen at 37°C for 1 hr. Non-adherent cells were removed and the remaining cells were cultured in 5 ml of supplemented RPMI and B6 lystes. B6 lystes were prepared by doing a single-cell suspension of spleen cells from naïve B6 mice and resuspended at $3x10^7$ cells/ml. This suspension was then frozen at -80°C for 15 minutes and thawed at 37°C for 10 minutes for one cycle. The suspension was then sonicated with 3 x 5 second pulsations. Lysates were incubated with BALB/c APC at 37°C overnight. BALB/c APC pulsed with B6 alloantigen were used in indirect mixed lymphocyte reactions (MLR), cytotoxic T lymphocyte (CTL) assays, and local adoptive transfer (LAT) assays. For B6 APC, spleen cells from naïve B6 mice were resuspended at $2x10^6$ cells/ml, incubated with 400 μg/ml of mitomycin-C for 1 hr at 37°C, and subsequently washed 3X with HBSS. APC were resuspended at $1x10^7$ cells/ml for MLR, $6x10^5$ cells/ml for CTL, and $5x10^7$ cells/ml for LAT assay.

Cytotoxic T lymphocyte (CTL) assay

A standard 4-h ^{51}Cr-release assay was used as previously described [24]. Briefly, single-cell suspensions of spleen cells were prepared from BALB/c mice with and without allergic conjunctivitis as well as mice with pre-vascularized graft beds 1-7 days after they rejected their B6 corneal allografts. $3x10^7$ splenocytes were suspended in supplemented RPMI medium and stimulated with BALB/c APC pulsed with B6 alloantigen or using B6 APC for 4-5 days. These splenocytes were then incubated *in vitro* in 96 well plates (Corning Inc., Corning, NY) along with 2×10^4 ^{51}Cr-labeled B16LS9HG tumor cells in a total volume of 200 μl/well for 4h. Assays were performed in triplicate using an effector to target cell ratio of 50:1. 100 ul of the

supernatant from each well was then collected and counted in a gamma counter (Packard BioScience, Meriden, CT). Cytotoxicity was determined by the amount of ^{51}Cr released by the target cells and the specific lysis was calculated as follows: % specific lysis = [(experimental cpm − spontaneous cpm) / (maximum release cpm − spontaneous release)] x 100.

Mixed Lymphocyte Reaction (MLR)

$CD4^+$ T cells from acceptors, rejectors, and allergic rejectors were isolated using the mouse CD4 isolation kit (Miltenyi Biotec). Purified $CD4^+$ T cells were harvested 4-7 days after rejection and incubated at 1 x 10^6 per well with respective APC at a 1:1 ratio in a total volume of 200 µl for 78 h and pulsed with [^3H] thymidine and incubated for an additional 18 hours. Incorporation of [^3H] thymidine was measured using a liquid scintillation counter.

***In vitro* suppression assay**

$CD4^+CD25^+$ Tregs were collected from spleens of cornea grafted mice 3 wk post-transplantation using Treg isolation kits (Miltenyi Biotec). A total of $5x10^4$ $CD4^+CD25^+$ Tregs isolated from corneal allograft acceptors or allergic rejectors were incubated in round bottom, 96-well plates with $1x10^5$ $CD4^+$ T effector cells from naïve WT mice or IL-4R$\alpha^{-/-}$ mice. Tregs were treated with mitomycin-C (400 µg/ml) for 30 minutes and then washed 3X with HBSS. The cells were stimulated *in vitro* with 1 mg/ml anti-CD3ε Ab (BD Biosciences) for 78 hours and then pulsed with [^3H] thymidine and incubated for an additional 18 hours. Incorporation of [^3H] thymidine was measured using a liquid scintillation counter. % suppression = [(Teff cpm) – (Teff + Tregs cpm) / (Teff cpm)] x 100. For experiments where rIL-4, rIL-5, or rIL-13 were added, co-cultures were supplemented with 25 ng/ml of the cytokine.

Local adoptive transfer (LAT) assay

$CD4^+CD25^+$ Tregs were mixed with BALB/c APC pulsed with B6 splenocytes and effector $CD4^+$ T cells from corneal allograft rejectors in a 1:1:1 ratio. Left and right ear pinnae of naive BALB/c mice were injected with 20 uL (1×10^6) of the mixed-cell population. The opposite ear was injected with HBSS as a negative control. Ear swelling was measured 24 hours later to assess DTH.

Statistical Analysis

The log-rank test was used for statistical analysis of the differences in the tempo of corneal graft rejection from the Kaplan–Meier survival curves. Clinical scores and ELISA data are represented as the mean ± SD. Inflammatory cell counts are represented as mean ± SE. Comparison between the WT immunized and knockout immunized mice were made using Student's t-test [183]. Significance of the histological data was tested by Student's t-test. *P* values of less than 0.05 were considered significant.

CHAPTER THREE

RESULTS

ALLERGIC CONJUNCTIVITIS

Model of Allergic Conjunctivitis

Allergic conjunctivitis is an ocular inflammatory disease that affects the lid, conjunctiva, and, in severe forms, the cornea [184]. It is estimated that allergic conjunctivitis affects 20% of the world's population [185-187]. A mild form of allergic conjunctivitis, known as seasonal allergic conjunctivitis, is induced by environmental irritants such as grass, ragweed, and birch pollens [186]. More severe forms, such as atopic keratoconjunctivitis or vernal keratoconjunctivitis, display more chronic symptoms and can involve the cornea [100, 188]. In order to understand the possible mechanism by which allergic diseases exacerbate graft rejection, our first aim was to optimize the protocol used to induce allergic conjunctivitis by Magone and colleagues [183].

Allergic conjunctivitis was induced by i.p. sensitization with SRW pollen in alum followed by topical challenges with SRW pollen administered to the right eye for seven consecutive days (Figure 3A) [189]. The early phase of allergic conjunctivitis was assessed by evaluating the clinical phenotype of the disease following daily topical challenge on the eye with SRW pollen and by scoring tear production, lid edema, chemosis, and conjunctival vasodilatation [183]. As mentioned previously, allergic conjunctivitis also consists of a late phase that is characterized by the infiltration of inflammatory cells consisting primarily of eosinophils and occurs six to twelve hours after allergen challenge. Accordingly, the late phase was assessed by evaluating eosinophillic infiltration into the conjunctiva by histological analysis.

In optimizing our protocol we found that SRW pollen induced a better expression of the clinical phenotype compared to SRW extract. Interestingly, both the extract and pollen induced similar eosinophillic infiltration into the conjunctiva. Based on these results, we decided to use SRW pollen for our allergic conjunctivitis study. WT BALB/c mice immunized and challenged with SRW pollen had a higher expression of the clinical phenotype of allergic conjunctivitis compared to the PBS controls (Figure 3B). Also, SRW pollen sensitization of WT BALB/c mice increased the number of eosinophils infiltrating into the conjunctiva compared to PBS controls (Figure 3C).

In examining the development of allergic conjunctivitis, we first wanted to determine and characterize the role of NKT and γδ T cells in the development of SRW pollen-induced allergic conjunctivitis. The first part of this study examined the hypothesis that as in allergic AHR, NKT and γδ T cells are required for maximal expression of allergic conjunctivitis.

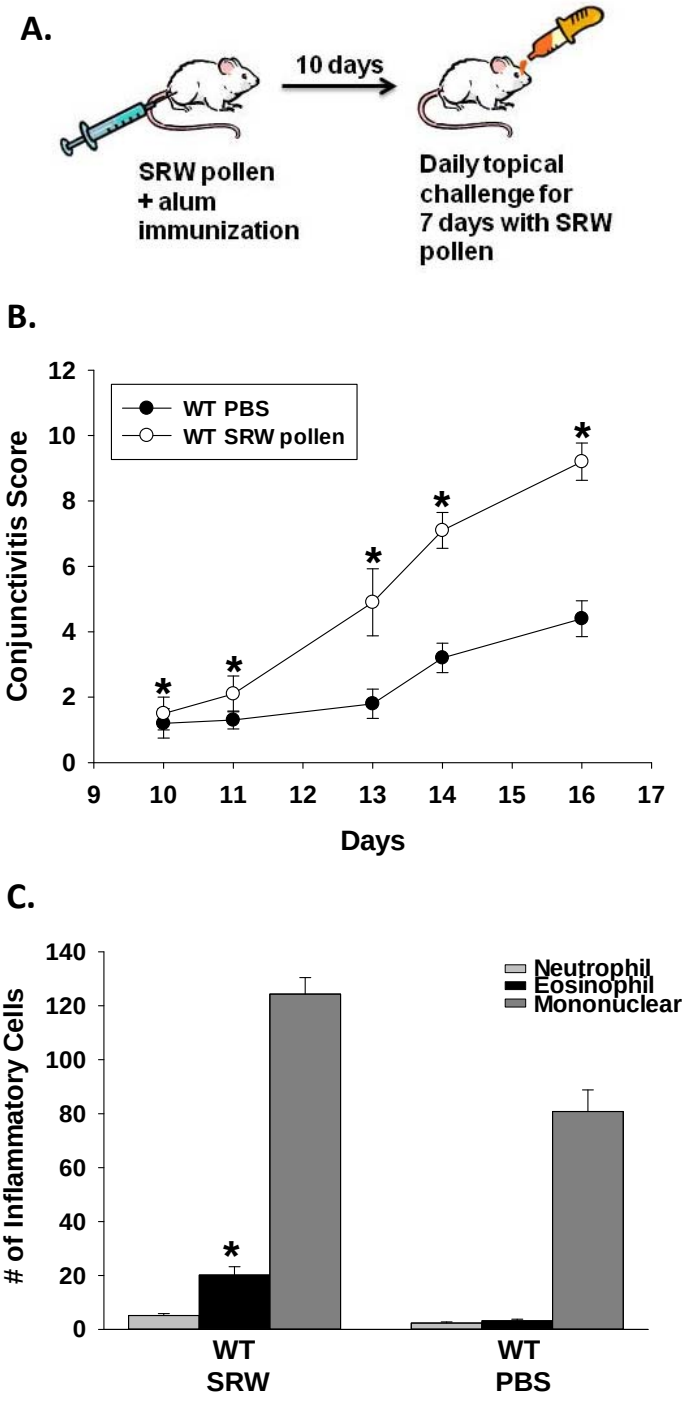


Figure 3. Allergic Conjunctivitis Model. A) Mice were immunized i.p. with SRW pollen in alum and ten days later were challenged topically for seven days. B) Clinical scores for allergic conjunctivitis in WT BALB/c mice sensitized with SRW pollen (○) or PBS control (●). C) Eosinophilic infiltration into the conjunctivae of WT BALB/c SRW pollen sensitized mice and PBS controls. Graphs are representative of three independent experiments
(n = 6 mice/group/experiment); * $p < 0.05$

NKT cells are required for the early and late phases of allergic conjunctivitis

The possibility that NKT cells were required for the maximal expression of allergic conjunctivitis was examined first. WT BALB/c mice were treated with either anti-CD1d monoclonal antibody or an isotype control antibody. WT BALB/c mice treated with anti-CD1d antibody had a decreased expression of the early phase of allergic conjunctivitis compared to the isotype control antibody-treated group (Figure 4A). There was also a decrease in the percent of infiltrating eosinophils into the conjunctiva in anti-CD1d-treated mice (Figure 4B). Experiments were also performed in Th-1 prone C57BL/6 (B6) and yielded similar results (data not shown).

Initial experiments in WT mice treated with anti-CD1d monoclonal antibody suggested that NKT cells might be required to elicit maximal expression of allergic conjunctivitis. To further explore the role of NKT cells in allergic conjunctivitis, we assessed the clinical expression of allergic conjunctivitis in $CD1d^{-/-}$ mice which lack type I and type II NKT cells. $CD1d^{-/-}$ BALB/c mice had decreased tear production, lid edema, chemosis, and conjunctival vasodilatation compared to WT mice (Figure 5A). Eosinophilic infiltration into the conjunctivae of $CD1d^{-/-}$ BALB/c mice was significantly decreased compared to the WT counterparts (Figure 5B). Experiments were also performed in $CD1d^{-/-}$ B6 mice with similar results (data not shown). Thus, type I and type II NKT cells are required to elicit the early and late phase of allergic conjunctivitis.

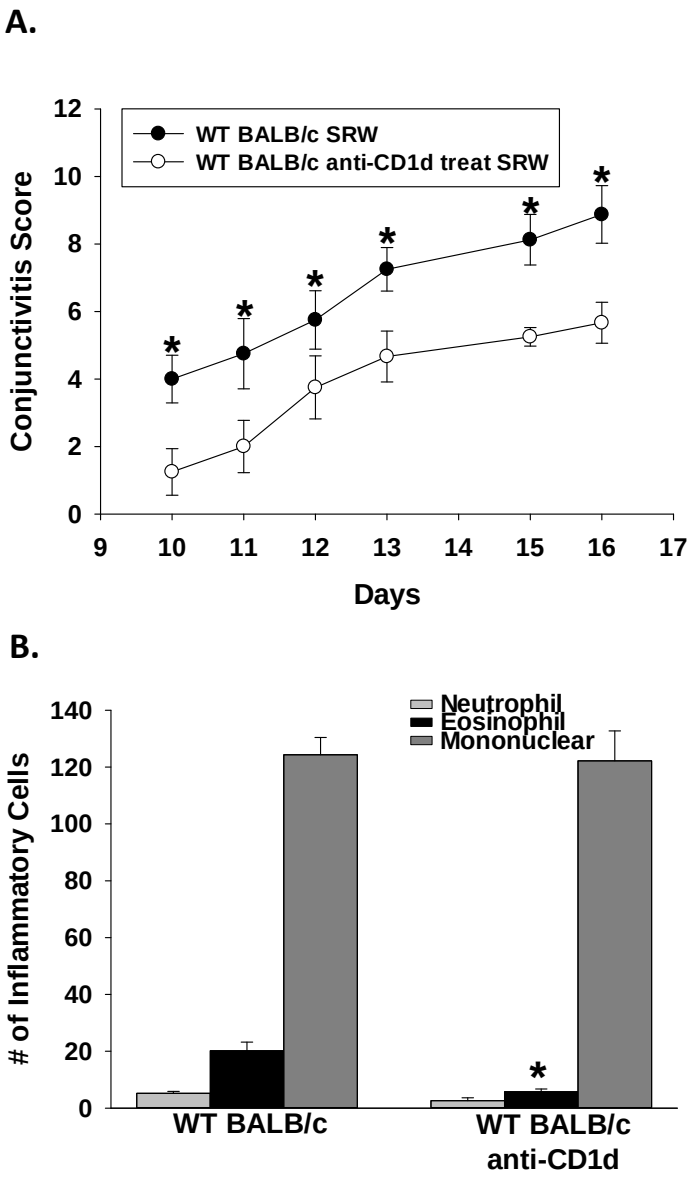


Figure 4. Blocking NKT cell activation results in decreased expression of allergic conjunctivitis. Clinical allergic conjunctivitis scores in BALB/c mice treated with either anti-CD1d antibody (○) or an isotype control antibody (●) prior to sensitization and challenge with SRW pollen. B) Eosinophilic infiltrations into the conjunctivae of SRW-pollen challenged mice. Graph is representative of three independent replicate experiments (n=5 mice/group/experiment); * $p < 0.05$

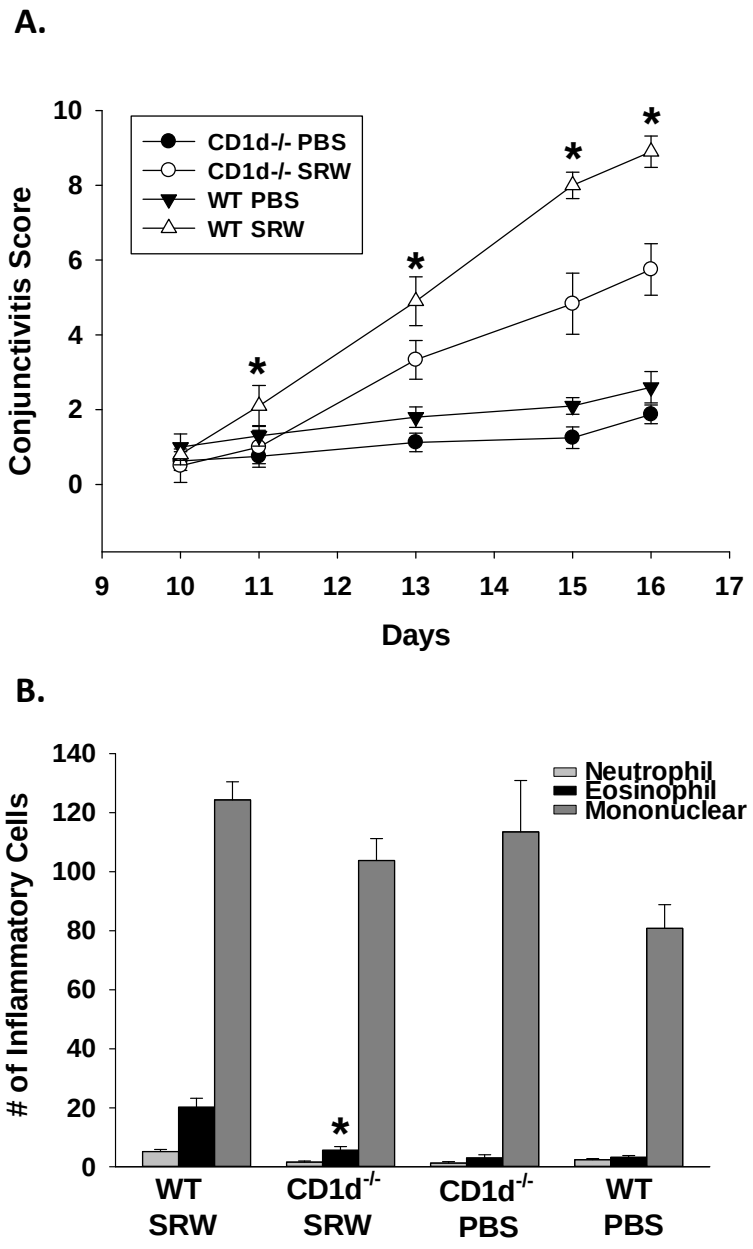


Figure 5. Type I and II NKT cells are required for the early and late phase of allergic conjunctivitis. A) Clinical scores for allergic conjunctivitis in $CD1d^{-/-}$ BALB/c mice (▼) and WT BALB/c mice (Δ) that were sensitized and challenged topically with SRW pollen. $CD1d^{-/-}$ BALB/c mice (●) and WT BALB/c mice (○) were challenged topically with PBS and served as negative controls. B) Eosinophilic infiltration into the conjunctivae of $CD1d^{-/-}$ BALB/c mice and WT BALB/c mice. Graph is representative of two independent experiments (n = 5 mice/group/experiment); * $p < 0.05$

Type I NKT cells are required for the early and late phases of allergic conjunctivitis

To determine if type I NKT cells are needed for allergic conjunctivitis, Jα18$^{-/-}$ mice were utilized because they lack only type I NKT cells. Jα18$^{-/-}$ BALB/c mice displayed a decrease in the clinical expression of allergic conjunctivitis when compared to WT mice which confirmed that type I NKT cells are required for the early phase of allergic conjunctivitis (Figure 6A). Compared to CD1d$^{-/-}$ mice, Jα18$^{-/-}$ mice expressed more severe allergic conjunctivitis, suggesting that there is an additive effect in the reduction of allergic conjunctivitis when both type I and type II NKT cells are missing. Histological analysis demonstrated that the number of eosinophils infiltrating into the conjunctivae of Jα18$^{-/-}$ BALB/c mice was significantly decreased compared to their WT counterpart (Figure 6B). Similar results were found using Jα18$^{-/-}$ B6 mice (data not shown). These results indicate that NKT-deficient mice in both Th1- and Th2-prone strains have diminished early phase reactions, as defined by the clinical scores as well as the late phase reactions as demonstrated by a decreased eosinophilic infiltration into the conjunctiva.

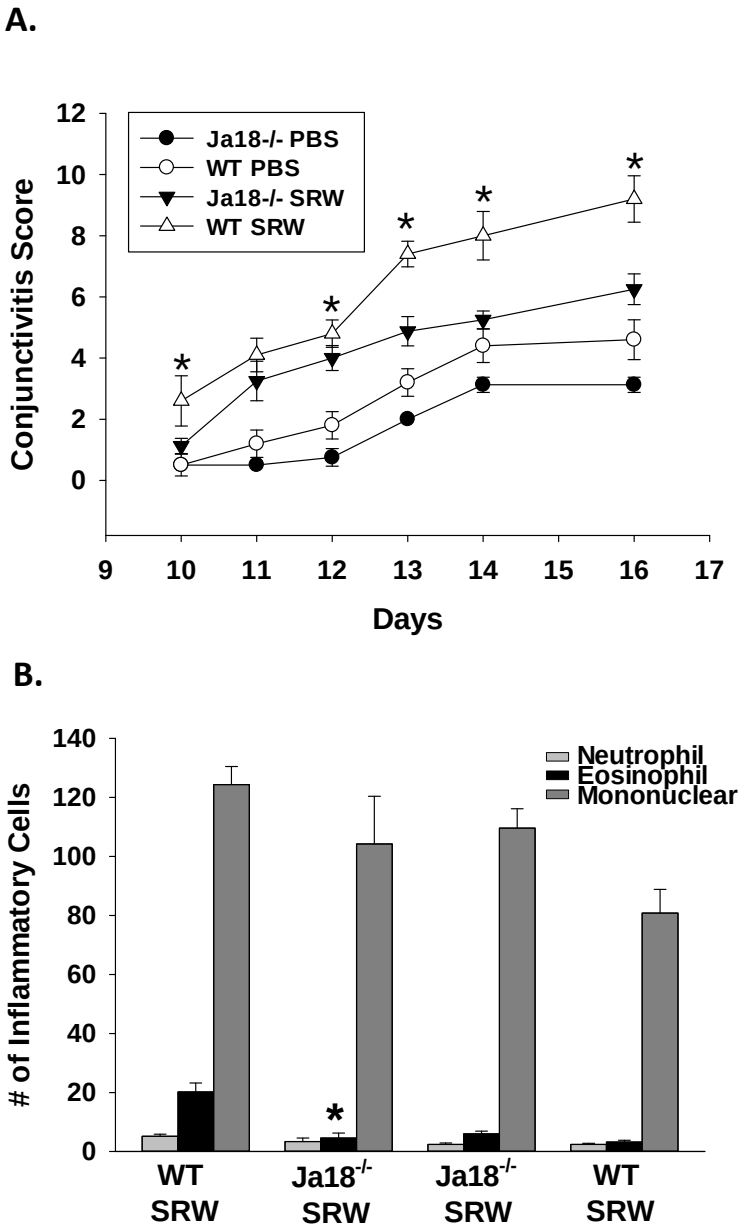


Figure 6. Type I NKT cells are required for the early and late phase of allergic conjunctivitis. A) Clinical scores of Jα18$^{-/-}$ BALB/c (▼) and WT BALB/c mice (Δ) that were sensitized and challenged with SRW pollen compared to Jα18$^{-/-}$ mice. Jα18$^{-/-}$ BALB/c mice (●) and WT BALB/c mice (○) were challenged with PBS and served as negative controls. B) Eosinophilic infiltration into the conjunctivae of Jα18$^{-/-}$ BALB/c and WT BALB/c mice. Graph is representative of three independent replicate experiments (n=5 mice/group/experiment);
* $p < 0.05$

Reconstituting NKT cells in NKT deficient mice restores allergic conjunctivitis

To confirm that the absence of NKT cells was responsible for the reduction of allergic conjunctivitis in Jα18$^{-/-}$ B6 mice, we reconstituted Jα18$^{-/-}$ B6 mice with 2.0 – 4.5 x 10^5 NKT cells isolated from naïve WT mice the day of immunization with SRW pollen. Adoptive transfer of cells double positive for NK1.1 and TCR-β fully restored the ability of Jα18$^{-/-}$ B6 mice to develop the early phase of allergic conjunctivitis (Figure 7A). When compared to WT B6 mice treated with SRW pollen, Jα18$^{-/-}$ B6 mice reconstituted with NKT cells had comparable numbers of eosinophils infiltrating into the conjunctiva (Figure 7B). T cells expressing NK1.1 and TCR-β were not required for maximal expression of the late phase of allergic conjunctivitis since spleen cells depleted of NK1.1 and TCR-β double positive cells also had comparable numbers of eosinophils when compared to WT B6 mice (Figure 7B). Since Jα18$^{-/-}$ mice lack NKT cells and were otherwise identical to WT B6 mice, these results verified that NKT cells are required for the development of allergic conjunctivitis.

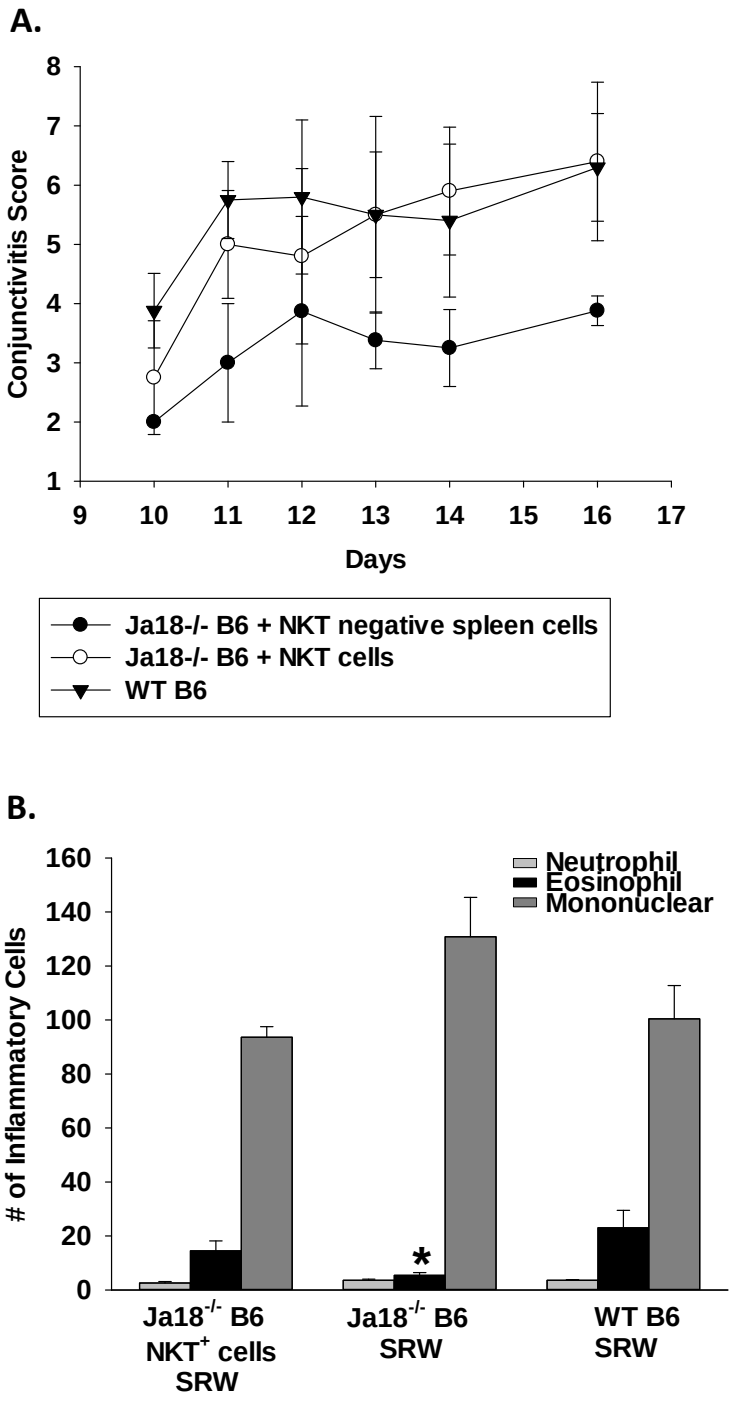


Figure 7. Adoptive transfer of NKT cells into Jα18$^{-/-}$ B6 mice restores allergic conjunctivitis. A) Clinical score of Jα18$^{-/-}$ B6 mice reconstituted with NKT positive cells (●), Jα18$^{-/-}$ B6 mice reconstituted with NKT negative spleen cells (○), and WT B6 mice (▼). B) Eosinophilic infiltration into the conjunctivae of Jα18$^{-/-}$ B6 mice reconstituted with NKT cells. Graph is representative of three independent replicate experiments (n=5 mice/group/experiment); * $p < 0.05$

γδT cells are required for the full expression of the early and late phase of allergic conjunctivitis

Next, we wanted to determine the role of γδT cells in allergic conjunctivitis. Allergic conjunctivitis was induced in TCR-$\delta^{-/-}$ and WT B6 mice and the early phase was assessed as previously described. Control mice for each group were challenged with PBS. The results demonstrate that TCR-$\delta^{-/-}$ mice had a reduction in the early phase of allergic conjunctivitis when compared to WT B6 mice (Figure 8A). The late phase of allergic conjunctivitis was assessed by evaluating the presence of inflammatory cells in the conjunctivae of WT and TCR-$\delta^{-/-}$ mice by histological analysis. SRW pollen-sensitized TCR-$\delta^{-/-}$ mice had less eosinophilic infiltration in the conjunctivae compared to WT mice, indicating that full expression of the late phase of allergic conjunctivitis requires the participation of γδ T cells (Figure 8B). To confirm the role of γδ T cells in allergic conjunctivitis, WT mice were treated with GL3 antibody, which inhibits the function of γδ T cells by blocking the TCRδ chain [190]. GL3 or hamster IgG isotype control antibody was administered twice a week beginning five days prior to immunization. WT mice treated with GL3 had a reduction in the clinical manifestations of allergic conjunctivitis as well as in the eosinophilic infiltration into the conjunctivae (Figure 9). Together, these results indicate that γδ T cells are required for the full expression of both the clinical manifestation and late phases of allergic conjunctivitis.

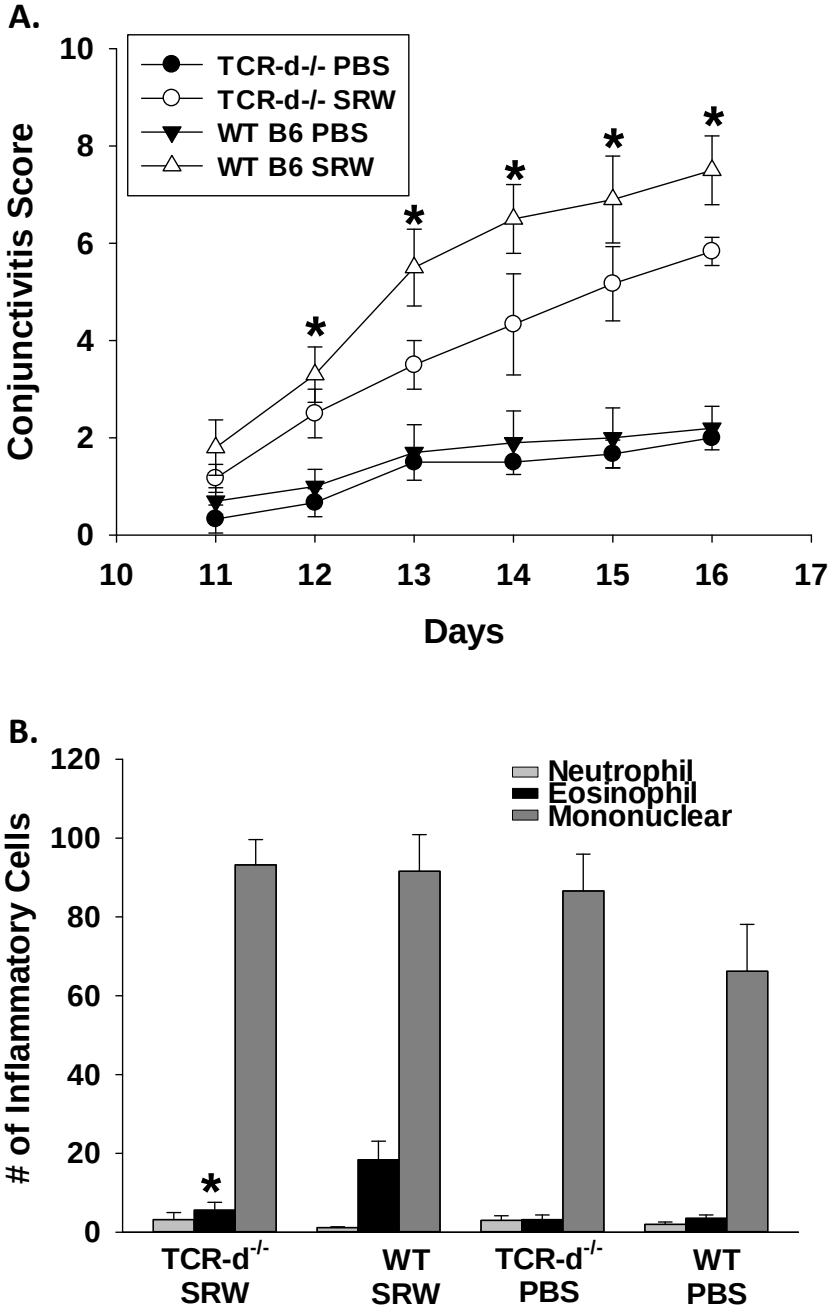


Figure 8. γδ T cells are required for the early and late phase of allergic conjunctivitis. A) Clinical scores for allergic conjunctivitis in TCR-$\delta^{-/-}$ B6 mice (○) and WT B6 mice (Δ) that were sensitized and challenged topically with SRW pollen. TCR-$\delta^{-/-}$ mice (●) and WT B6 mice (▼) were challenged topically with PBS and served as negative controls. B) Eosinophilic infiltration into the conjunctivae of TCR-$\delta^{-/-}$ B6 mice and WT B6 mice. Graph is representative of two independent experiments (n=5 mice/group/experiment); * $p < 0.05$

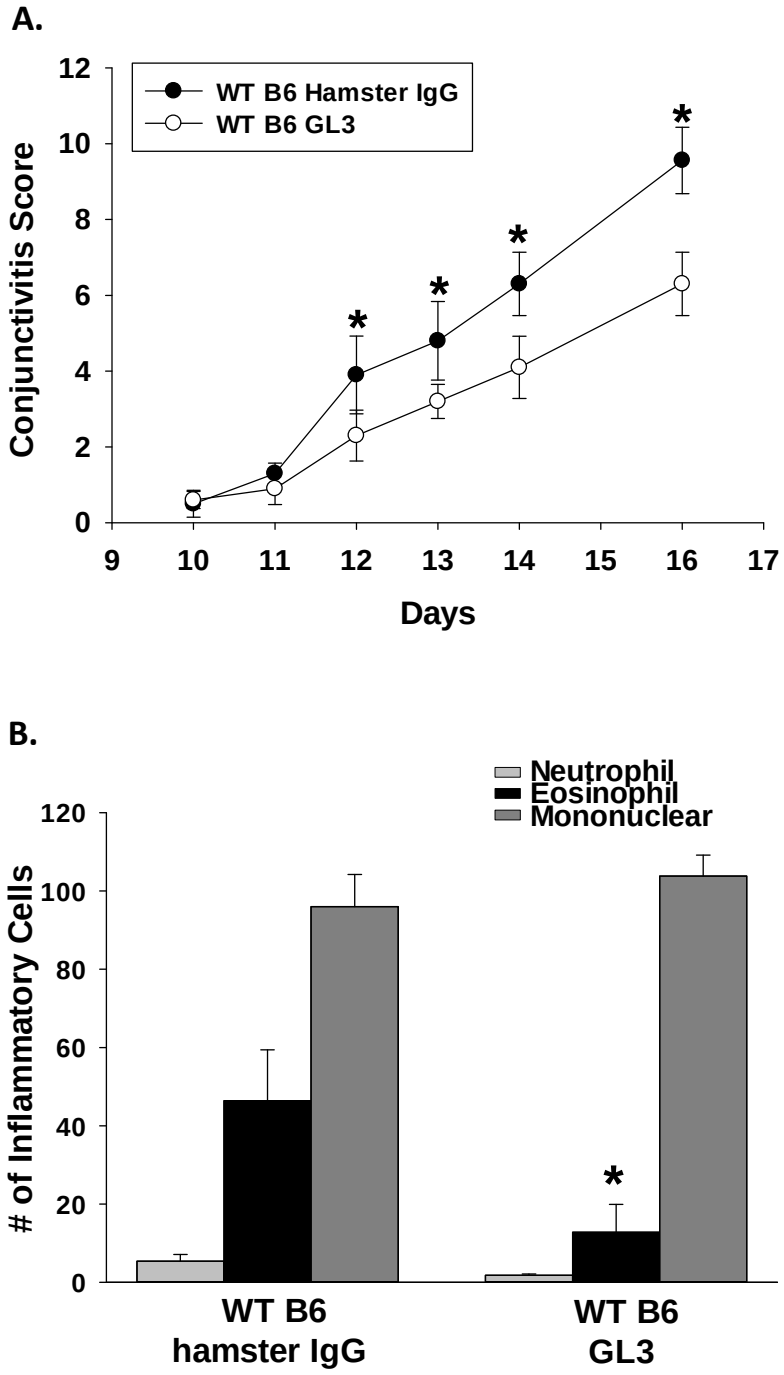


Figure 9. Inhibiting the function of γδ T cells causes a reduction in allergic conjunctivitis. A) Clinical scores for allergic conjunctivitis in WT B6 mice treated with GL3 antibody (○) or hamster IgG antibody (●) that were sensitized and challenged topically with SRW pollen. B) Eosinophilic infiltration into the conjunctivae of WT B6 mice treated with GL3 antibody or hamster IgG antibody. Graph is representative of two independent experiments (n=5 mice/group/experiment); * $p < 0.05$

Effect of disabling NKT cells in TCR-δ$^{-/-}$ mice on allergic conjunctivitis

We have shown that NKT cells and γδ T cells are needed for maximal expression of allergic conjunctivitis [189, 191]. Therefore, we next wanted to determine what effect disabling NKT cells in γδ T cell-deficient mice would have on allergic conjunctivitis. Accordingly, TCR-δ$^{-/-}$ mice were treated with anti-CD1d antibody three times a week beginning seven days prior to immunization with SRW pollen.

As previously shown, WT mice treated with anti-CD1d had a reduction in the clinical manifestation of the disease when compared to WT mice treated with isotype control antibody. Interestingly, TCR-δ$^{-/-}$ mice treated with anti-CD1d had a further reduction in the clinical features of allergic conjunctivitis compared to TCR-δ$^{-/-}$ mice treated with isotype control antibody (Figure 10A). This suggests that NKT cells and γδ T cells cooperate to promote full induction of allergic conjunctivitis. When assessing the late phase of allergic conjunctivitis, eosinophilic infiltration into the conjunctiva of anti-CD1d-treated TCR-δ$^{-/-}$ mice was comparable to TCR-δ$^{-/-}$ mice (Figure 10B). These results indicate that disabling NKT cells in γδ T cell-deficient mice blunts the clinical manifestation of allergic conjunctivitis, but does not have an additive effect on the late phase inflammatory response.

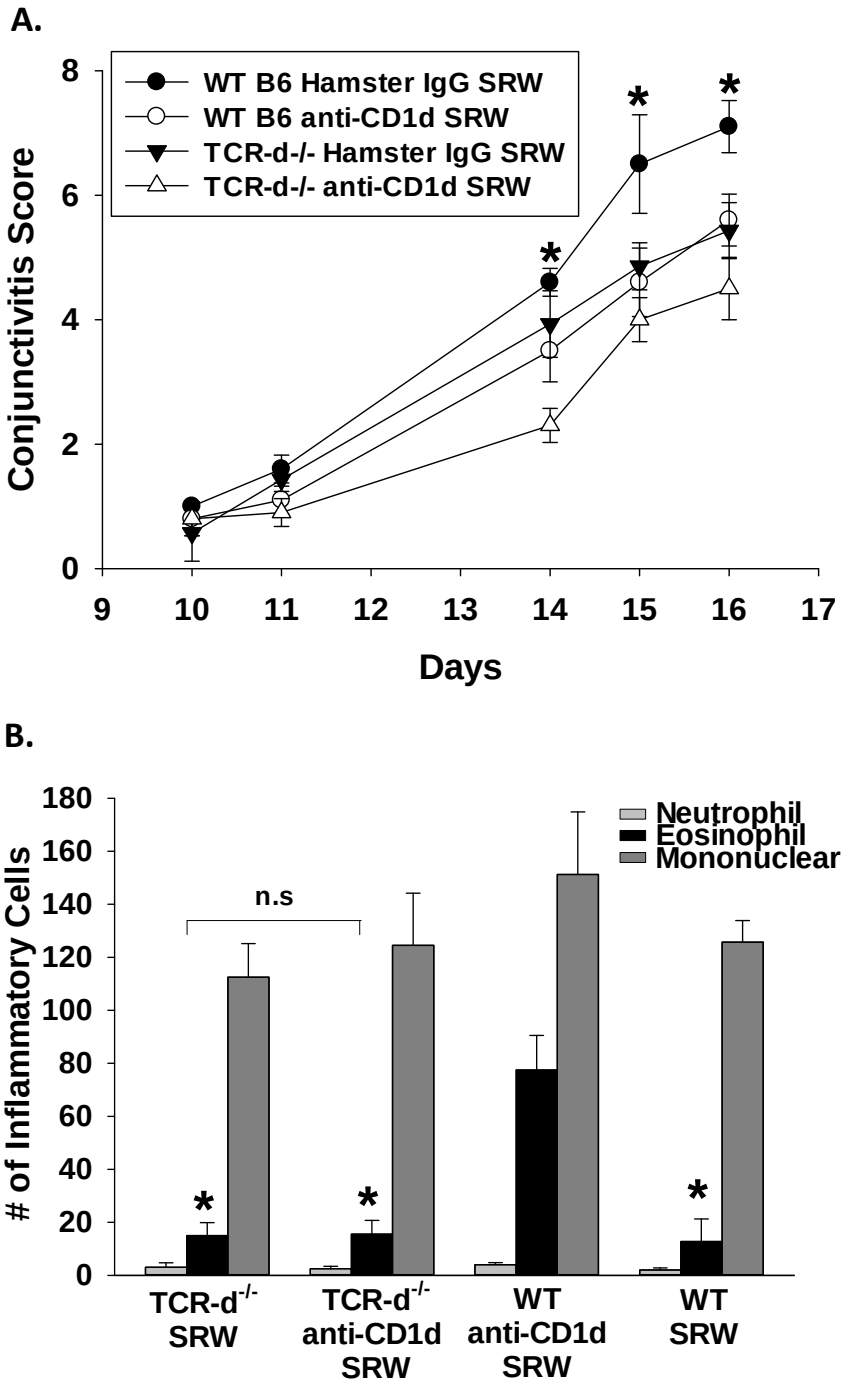


Figure 10. Blocking activation of NKT cells in γδ T cell-deficient mice further reduces allergic conjunctivitis. A) Clinical scores of allergic conjunctivitis in TCR-$\delta^{-/-}$ B6 mice treated with anti-CD1d antibody (Δ) or hamster IgG antibody (▼) as well at WT B6 mice treated with anti-CD1d (○) or hamster IgG antibody (●). B) Eosinophilic infiltration into the conjunctivae. Graph is representative of two independent experiments (n=5 mice/group/experiment); * $p < 0.05$

NKT cell-deficient mice have diminished production of Th2 cytokines

The production of Th2 cytokines (IL-4, IL-5, and IL-13) plays a critical role in the induction of allergic conjunctivitis [107, 192]. We examined the hypothesis that the reduction in the early phase and late phase reactions of allergic conjunctivitis was due to a decrease in cytokine production by SRW-specific Th2 cells. Splenocytes from SRW pollen-sensitized Jα18$^{-/-}$ BALB/c and WT BALB/c mice were stimulated *in vitro* with SRW extract for 48 hours and the presence of Th2 cytokines was quantified by ELISA.

Spleen cells from Jα18$^{-/-}$ BALB/c mice displayed a decreased production of Th2 cytokines IL-4, IL-5, and IL-13 compared to WT mice, while the production of the Th1 cytokine, IFN-γ, was comparable to WT levels (Figure 11). This suggests that type I NKT cells are necessary for optimal expression of allergic conjunctivitis.

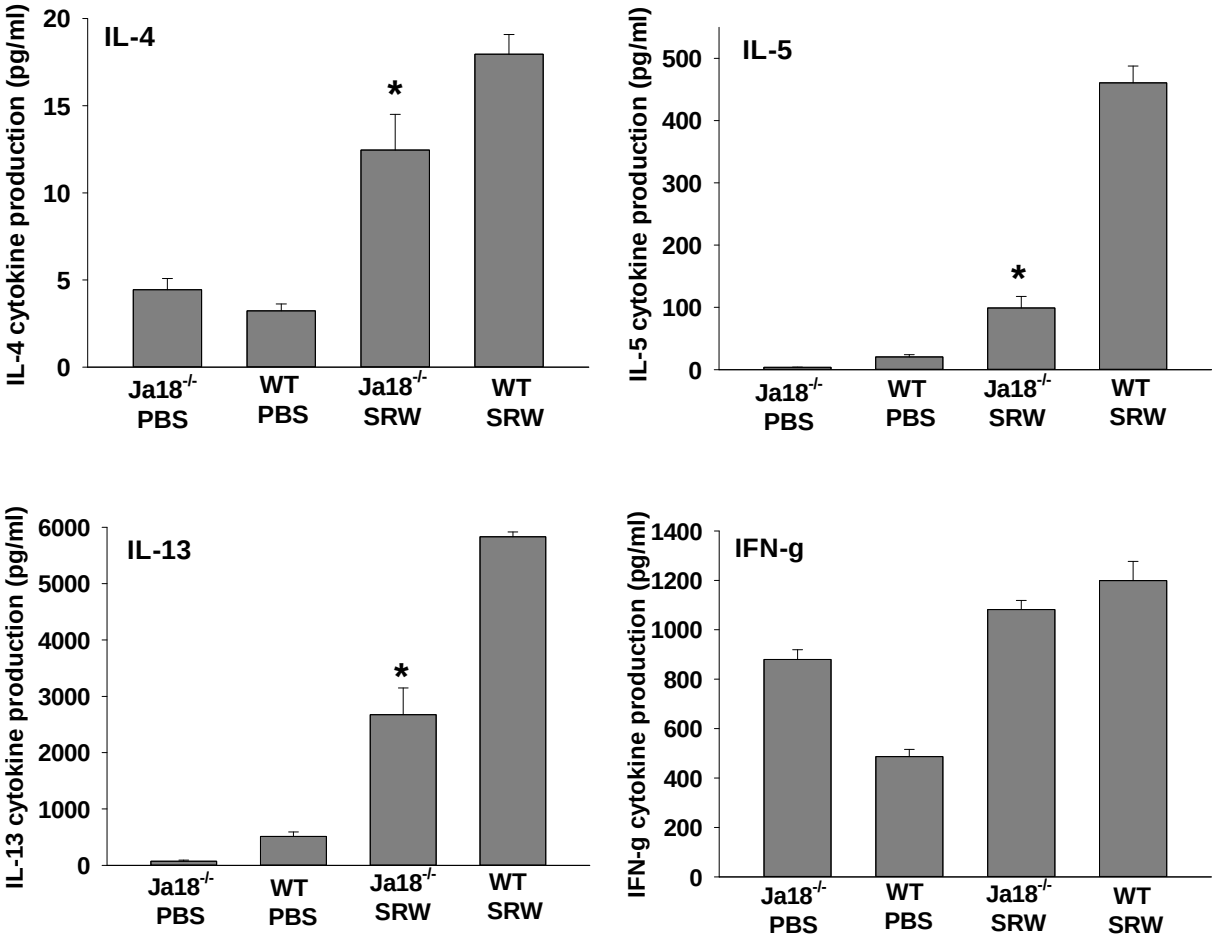


Figure 11. Decreased severity of allergic conjunctivitis in NKT cell-deficient mice is associated with diminished production of IL-4, IL-5, and IL-13. Bulk splenocytes were incubated with SRW extract. Supernatants were collected 48 hr after *in vitro* culture and analyzed by ELISA. Graphs are representative of two independent replicate experiments (n=5 mice/group/experiment); * $p < 0.05$

Production of Th2 cytokines in γδ T cell-deficient mice

We next assessed the hypothesis that the reduction in the clinical manifestations and late phase reactions of allergic conjunctivitis was due to a decrease in cytokine production by SRW-specific Th2 cells. As previously described, splenocytes from SRW pollen-sensitized TCR-$\delta^{-/-}$ B6 and WT B6 mice were stimulated *in vitro* with SRW extract for 48 hours and the production of Th2 cytokines was quantified by ELISA.

SRW-stimulated spleen cells from TCR-$\delta^{-/-}$ B6 mice displayed a decreased production of the Th2 cytokines IL-4, IL-5, and IL-13 compared to WT mice, while the production of the Th1 cytokine IFN-γ, was comparable to WT levels (Figure 12). This suggested that the presence of γδ T cells is necessary for optimal expression of allergic conjunctivitis.

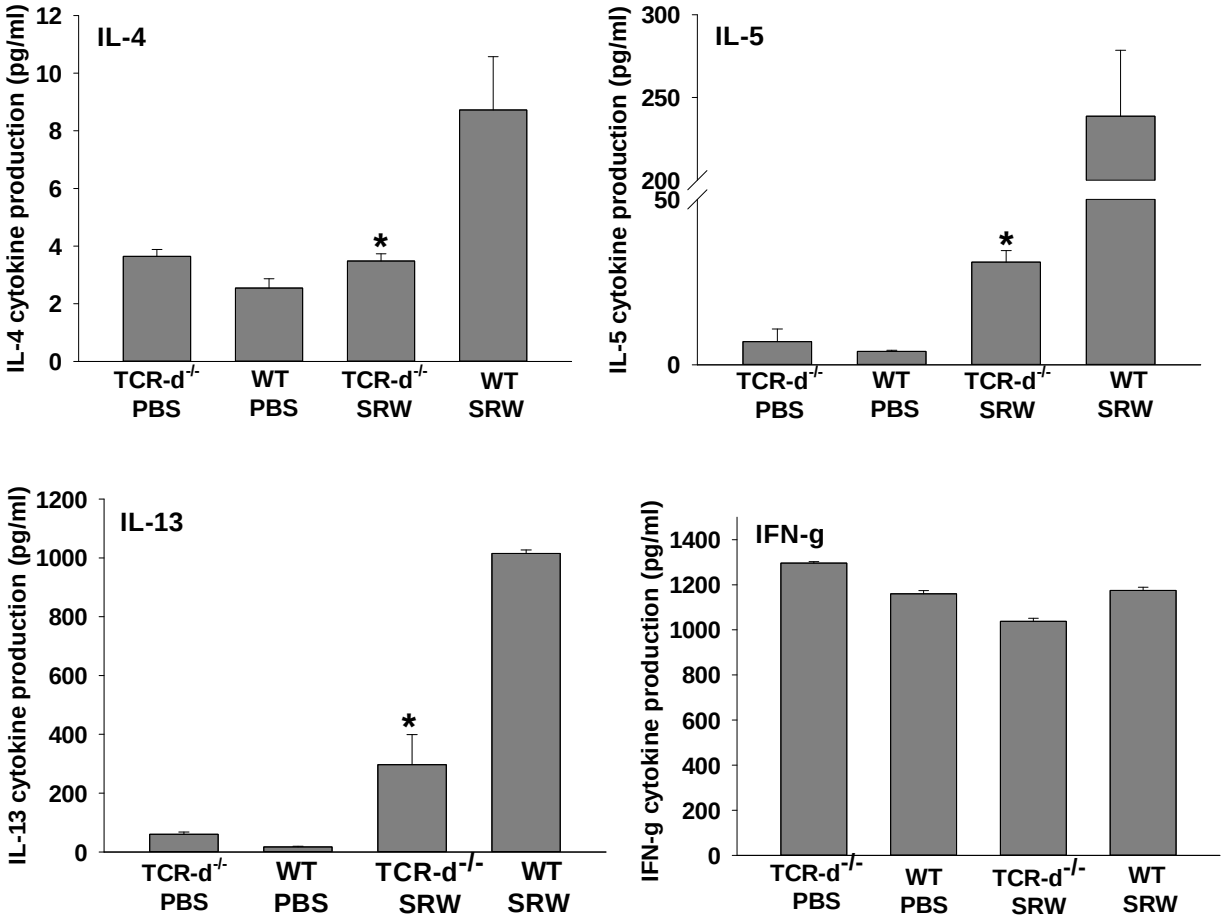


Figure 12. Decreased severity of allergic conjunctivitis in γδ T cell-deficient mice is associated with diminished production of IL-4, IL-5, and IL-13. Bulk splenocytes were incubated with SRW extract. Supernatants were collected 48 hr after *in vitro* culture and analyzed by ELISA. Graph is representative of two independent experiments (n=5 mice/group/experiment); * $p < 0.05$

NKT cells are necessary for the efferent arm of allergic conjunctivitis

Thus far, the results indicated that NKT cells were required for activating the afferent arm of the Th2 immune response as shown by the diminished production of Th2 cytokines that occurred in the Jα18$^{-/-}$ mice. Studies on allergic AHR in mice indicate that iNKT cells play an important role in the efferent arm of the Th2 immune response [119]. That is, iNKT cells in the lung produce significant quantities of IL-4 and IL-13, which create a Th2-biased milieu in the lung. Circulating Th2 cells that enter the lung are exposed to the Th2 cytokines and are boosted or "licensed" to produce Th2-based inflammation. In this situation, iNKT cells act on the efferent arm of the Th2 immune response by affecting the function of previously activated Th2 cells. With this in mind, adoptive transfer experiments were performed to first determine if iNKT cells might also influence the afferent arm of the immune response in allergic conjunctivitis. Accordingly, CD4^{+} T cells from SRW pollen-sensitized Jα18$^{-/-}$ BALB/c or WT BALB/c donors were adoptively transferred into naïve WT BALB/c recipients. Four days after the adoptive transfer, both groups of mice were challenged topically with SRW pollen daily for seven consecutive days. Both the clinical manifestation (early phase reaction of allergic conjunctivitis) and the number of inflammatory cells present in the conjunctiva (late phase reaction of allergic conjunctivitis) were assessed clinically and histopathology, respectively.

WT BALB/c naïve recipients of CD4^{+} T cells from Jα18$^{-/-}$ SRW pollen-sensitized donors displayed decreased clinical signs of allergic conjunctivitis compared to WT naïve recipients of CD4^{+} T cells from SRW pollen-sensitized WT donors (Figure 13A). The same trend was seen in B6 mice. The reduced severity of allergic conjunctivitis was consistent with the previously observed diminished Th2 cytokine production by CD4^{+} T cells from the Jα18$^{-/-}$ mice. In contrast, the WT BALB/c recipients of CD4^{+} T cells from Jα18$^{-/-}$ donors had eosinophilic

infiltrations in the conjunctivae that were similar to WT recipients of $CD4^+$ T cells from WT donors (Figure 13B). These results suggest that NKT cells are needed for the maximal generation of Th2 cells and for the expression of the early phase reaction of allergic conjunctivitis. Th2 cells generated in the absence of NKT cells were capable of mediating late phase inflammation if an NKT cell repertoire was available to license this Th2 cell population in the conjunctiva. Together these data suggested that in order for $CD4^+$ T cells to mediate maximal expression of the early phase of allergic conjunctivitis, NKT cells needed to be present during the afferent arm of the immune response. By contrast, the same $CD4^+$ T cells generated in the absence of NKT cells could mediate the late phase reaction as long as an there was an NKT cell population present in the conjunctivae.

The ability of Th2 cells from $J\alpha18^{-/-}$ donor mice to produce late phase inflammation suggested that the previously observed diminution of allergic conjunctivitis in $J\alpha18^{-/-}$ mice sensitized with SRW pollen was due to impaired expression of Th2-based inflammation (i.e., efferent arm). This was tested by adoptively transferring $CD4^+$ T cells from SRW pollen-sensitized WT donors to $J\alpha18^{-/-}$ recipients and assessing both the early phase and late phase reactions. The results of this adoptive cell transfer experiment demonstrated that the absence of an intact NKT cell repertoire prevented full expression of both the early phase and late phase reactions of allergic conjunctivitis by the SRW pollen-sensitized $CD4^+$ T cells from WT mice (Figure 13C and 13D). These results suggested that NKT cells function at the end stage organ (i.e., conjunctiva) and, as in allergic AHR, may act to license Th2 cells in the conjunctiva for the maximal expression of allergic conjunctivitis by either regulating additional elements required for the development of allergic conjunctivitis or contributing to the magnitude of the Th2 immune response.

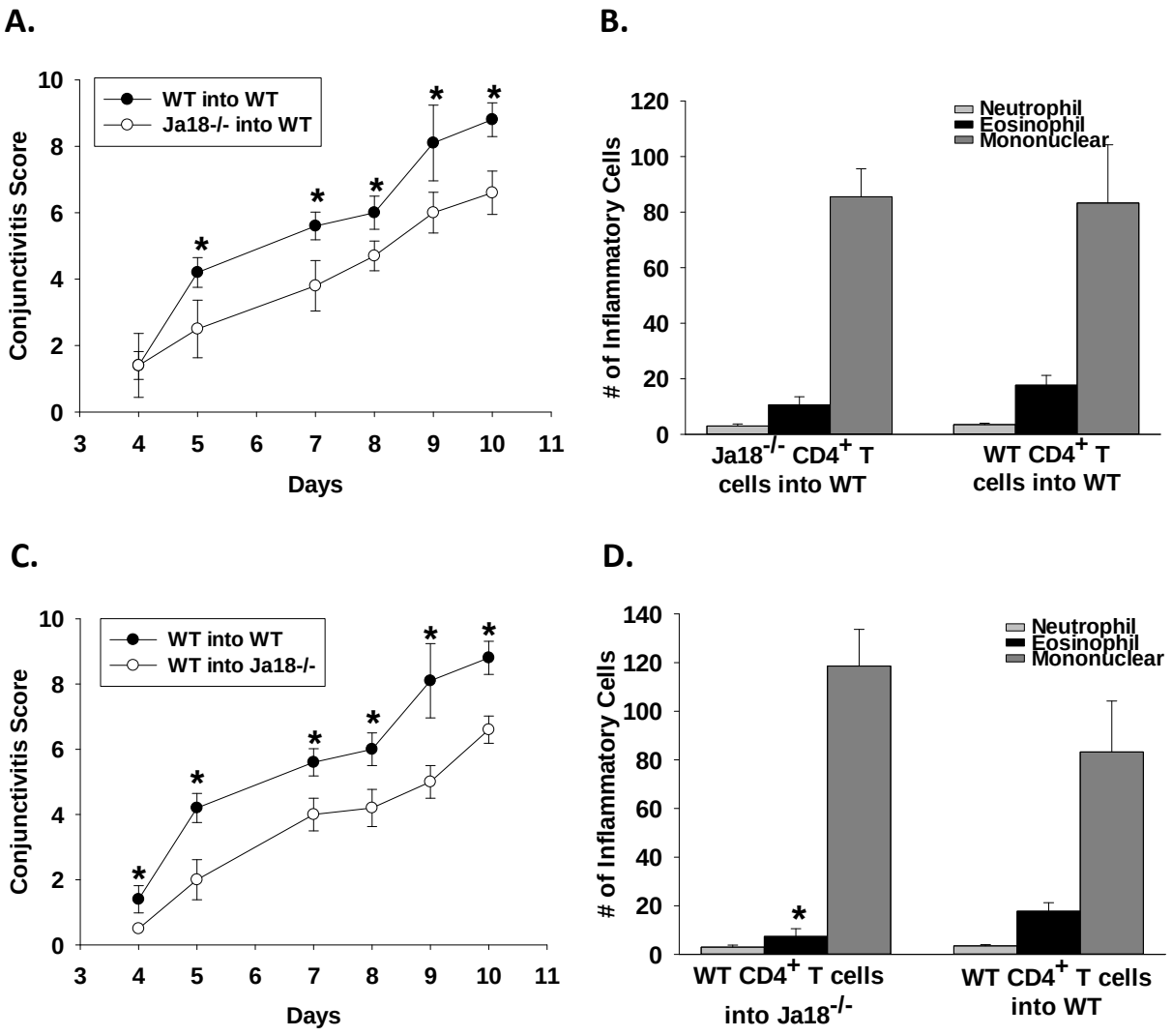


Figure 13. NKT cells participate in the afferent and efferent arms of allergic conjunctivitis. $CD4^+$ T cells isolated from SRW-sensitized donors were adoptively transferred into naïve recipients. A) Clinical scores of allergic conjunctivitis in naïve WT recipients that received $CD4^+$ T cells from either $J\alpha18^{-/-}$ donors (○) or WT donors (●) that were sensitized with SRW pollen. B) Eosinophilic infiltration into the conjunctivae of WT recipients of $CD4^+$ T cells from SRW-sensitized $J\alpha18^{-/-}$ or WT donors. C) Clinical scores of allergic conjunctivitis in naïve WT (●) or $J\alpha18^{-/-}$ (○) recipients of $CD4^+$ T cells from WT donors that were sensitized with SRW pollen. D) Eosinophilic infiltration into the conjunctivae of $J\alpha18^{-/-}$ and WT BALB/c mice that received $CD4^+$ T cells from WT BALB/c mice that were sensitized with SRW pollen. Graph is representative of two independent replicate experiments (n=5 mice/group/experiment); * $p < 0.05$

Role of γδ T cells in the afferent and efferent arms of allergic conjunctivitis

We also wanted to examine the effect γδ T cells have on the afferent and efferent arm of allergic conjunctivitis. The afferent phase involves APC capturing antigen and migrating to regional lymph nodes where they present antigen to T cells, which subsequently become activated, undergo clonal expansion, and differentiate into the various subtypes of T helper cells [193]. The antigen-specific T cells function as effector cells in the efferent phase of the immune response and, in the case of allergic conjunctivitis, enhance IgE antibody production, secrete cytokines, and participate in inflammation [99]. These results indicated that γδ T cells are required for activation of the afferent phase of the Th2 immune response as shown by the diminished production of Th2 cytokines that occurred in the TCR-$\delta^{-/-}$ B6 mice. Thus, it was hypothesized that γδ T cells were needed for the generation of SRW pollen-specific Th2 cells in the afferent phase of allergic conjunctivitis. To test this hypothesis, $CD4^+$ T cells from SRW pollen-sensitized TCR-$\delta^{-/-}$ or WT B6 donors were adoptively transferred into naïve WT B6 recipients. Four days after the adoptive transfer, both groups of mice were challenged topically with daily applications of SRW pollen for seven consecutive days. The clinical manifestation and the number of inflammatory cells present in the conjunctivae were assessed clinically and by histopathology respectively.

WT naïve recipients of $CD4^+$ T cells from TCR-$\delta^{-/-}$ B6 SRW pollen-sensitized donors displayed decreased clinical signs of allergic conjunctivitis compared to WT naïve recipients of $CD4^+$ T cells from SRW pollen-sensitized WT B6 donors. The reduced severity of allergic conjunctivitis was consistent with the previously observed diminished production of Th2 cytokines by $CD4^+$ T cells from the TCR-$\delta^{-/-}$ B6 mice (Figure 14A and 14B). In contrast, WT B6 recipients of $CD4^+$ T cells from TCR-$\delta^{-/-}$ B6 donors had granulocytic cell infiltration into the

conjunctivae that was similar to that found in WT B6 recipients of $CD4^+$ T cells from WT B6 donors, suggesting that the host's γδ T cell repertoire, presumably in the conjunctiva, can support the expression of the late phase of allergic conjunctivitis that is mediated by adoptively transferred Th2 cells. Together, these results suggest that γδ T cells are responsible for the maximal generation of Th2 cells and for the expression of the allergic conjunctivitis early phase reaction.

The capacity of Th2 cells from TCR-$\delta^{-/-}$ B6 donor mice to produce late phase inflammation in hosts with an intact γδ T cell repertoire suggested that the previously observed diminution of allergic conjunctivitis in TCR-$\delta^{-/-}$ B6 mice sensitized with SRW pollen was due to impaired expression of Th2-based inflammation in both the afferent and efferent arms of the immune response. It was also important to determine if γδ T cells affected the late phase of allergic conjunctivitis. This hypothesis was tested by adoptively transferring $CD4^+$ T cells from SRW pollen-sensitized WT B6 donors into TCR-$\delta^{-/-}$ B6 recipients and assessing both the clinical manifestations and late phase inflammation in allergic conjunctivitis. The results of this adoptive cell transfer experiment demonstrated that the absence of an intact γδ T cell repertoire prevented full expression of both the clinical expression and late phase inflammatory reactions of allergic conjunctivitis by SRW pollen-sensitized WT B6 $CD4^+$ T cells (Figure 14C and 14D). These results suggested that γδ T cells also function at the end stage organ (i.e., conjunctiva) and may be contributing to the magnitude of the Th2 immune response in the conjunctivae.

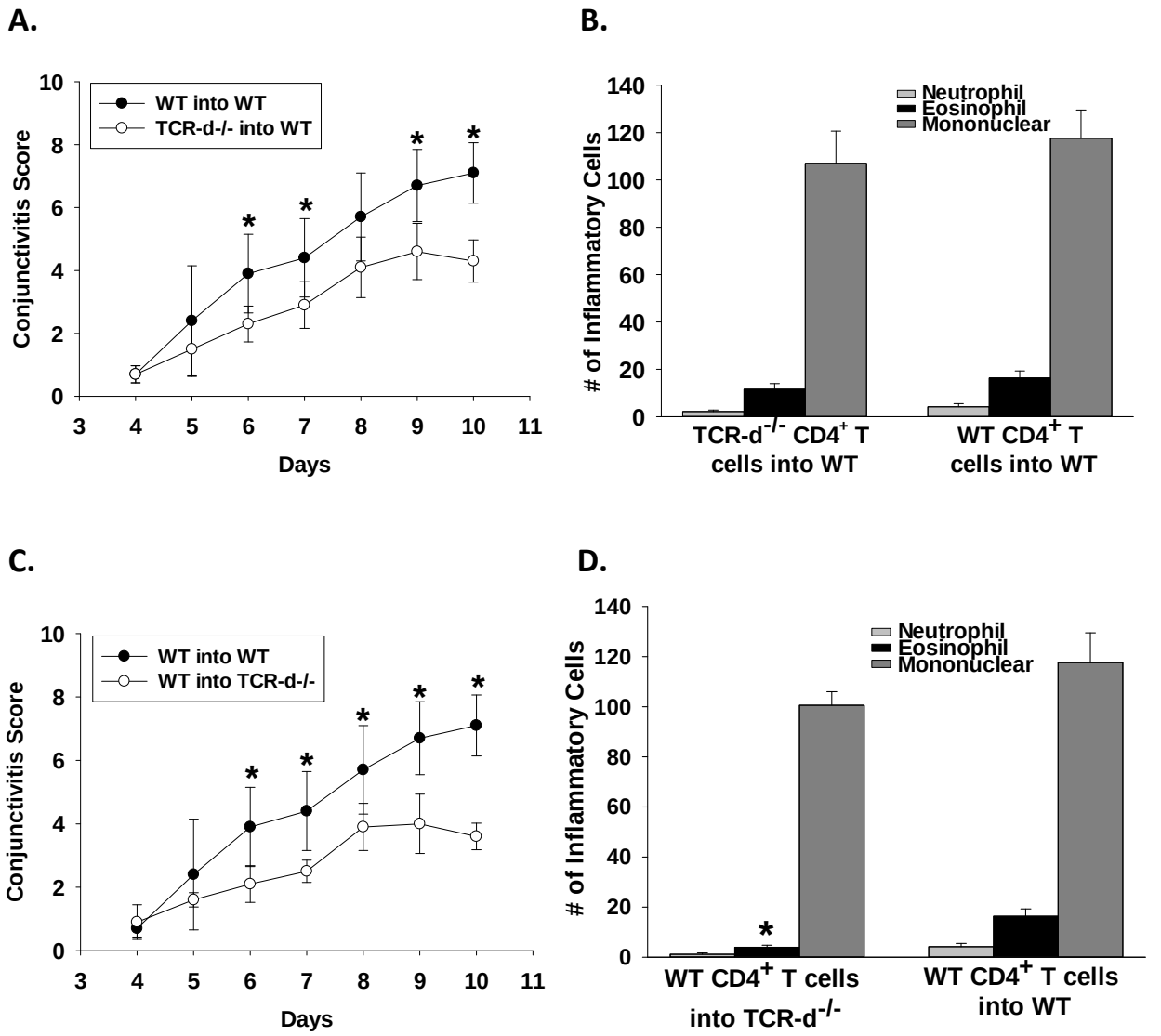


Figure 14. γδ T cells participate in the afferent and efferent arms of allergic conjunctivitis. $CD4^+$ T cells isolated from SRW-sensitized B6 donors were adoptively transferred into naïve recipients. A) Clinical scores of allergic conjunctivitis in naïve WT B6 recipients that received $CD4^+$ T cells from either WT B6 donors (●) or TCR-$\delta^{-/-}$ donors (○) or that were sensitized with SRW pollen. B) Eosinophilic infiltration into the conjunctivae of WT recipients of $CD4^+$ T cells from SRW-sensitized TCR-$\delta^{-/-}$ or WT B6 donors. C) Clinical scores of allergic conjunctivitis in naïve WT (●) or TCR-$\delta^{-/-}$ (○) recipients of $CD4^+$ T cells from WT B6 donors that were sensitized with SRW pollen. D) Eosinophilic infiltration into the conjunctivae of TCR-$\delta^{-/-}$ and WT B6 mice that received $CD4^+$ T cells from WT B6 mice that were sensitized with SRW pollen. Graph is representative of two independent replicate experiments (n=5 mice/group/experiment); * $p < 0.05$

Allergic Conjunctivitis and Corneal Allograft Rejection

Corneal allograft survival in mice with allergic conjunctivitis

Our laboratory uses a well established mouse model of keratoplasty. Corneal buttons from B6 mice are transplanted onto BALB/c recipient mice. The grafts are followed for a period of 60 days during which the opacity of the graft is scored on a scale of 0 to 3 (Figure 15A). Corneal allograft rejection in this model is determined after a graft receives two successive scores of 3. Within the 60 days, 50% of the animals experience graft rejection with a mean rejection time (MRT) of 35 ± 8 days and a median survival time (MST) of 52 days (Figure 16C).

Corneal allograft rejection is associated with the production of IFN-γ, IL-2, and alloantigen-specific DTH responses [153, 154]. We proposed that deviation of the immune response to a Th2 response by the induction of allergic conjunctivitis would increase corneal allograft survival. Accordingly, allergic conjunctivitis was induced as described earlier in BALB/c recipients prior to receiving a B6 corneal allograft (Figure 15B). This resulted in an increased incidence and swifter tempo of rejection (MRT of 17 ± 8 days and MST of 13) (Figure 16C).

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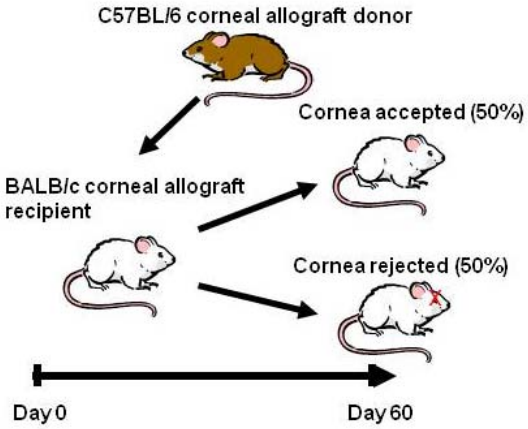
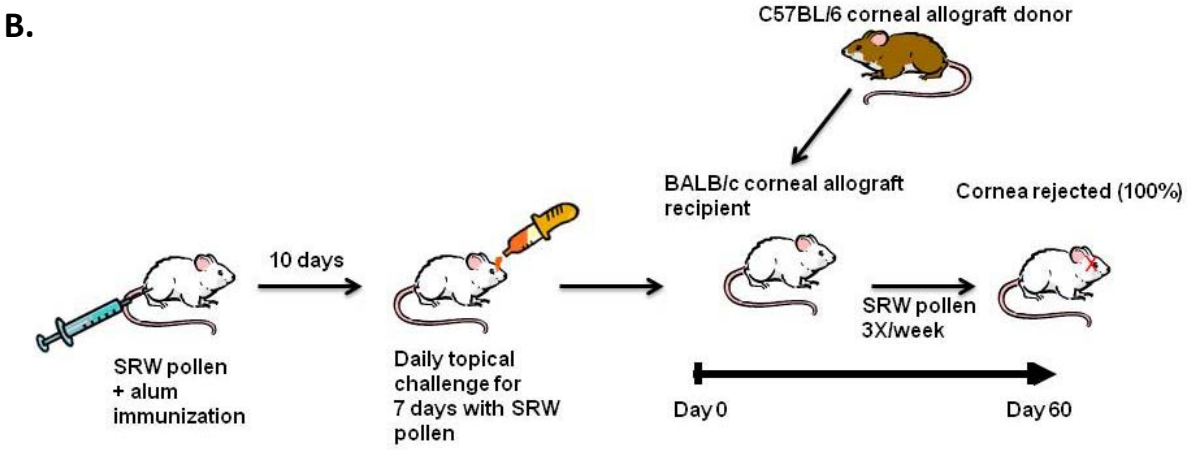
A.


C57BL/6 corneal allograft donor
Cornea accepted (50%)
BALB/c corneal allograft recipient
Cornea rejected (50%)
Day 0
Day 60

B.


C57BL/6 corneal allograft donor
BALB/c corneal allograft recipient
Cornea rejected (100%)
10 days
SRW pollen 3X/week
SRW pollen + alum immunization
Daily topical challenge for 7 days with SRW pollen
Day 0
Day 60

C.

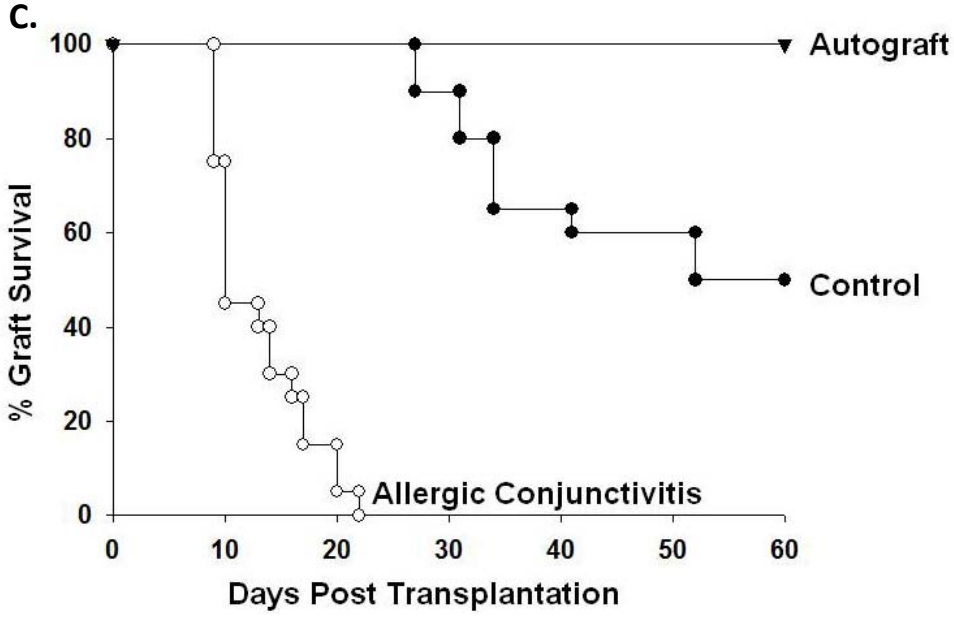

Autograft
Control
Allergic Conjunctivitis
% Graft Survival
Days Post Transplantation
0
20
40
60
80
100
0
10
20
30
40
50
60

Figure 15. Model of penetrating keratoplasty. A) Experimental design for murine keratoplasty in which BALB/c recipient mice receive orthotopic B6 corneal allografts. The transplanted corneas are followed for a period of 60 days. B) Allergic conjunctivitis is induced in BALB/c recipients prior to placing a B6 corneal allograft. C) Survival curve for a corneal allograft in which 50% of the corneal allografts will survive and 50% will undergo rejection. If the recipients have allergic conjunctivitis, rejection increases to 100%.

Allergic Conjunctivitis and ACAID induction

In the first aim of our study we wanted to determine if allergic conjunctivitis affected the induction and expression of ACAID. ACAID is an important mechanism influencing corneal allograft survival [59, 71-73]. Studies have shown that injection of donor cells into the AC of corneal allograft recipient mice before transplantation induces ACAID and results in a significant enhancement of corneal allograft survival [69, 70]. We hypothesized that allergic conjunctivitis inhibits the development of ACAID and therefore causes an increase in allograft rejection. To test this hypothesis, allergic conjunctivitis was induced through the topical application of SRW pollen in the right eye of BALB/c mice before AC injections of B6 spleen cells into the left eyes. After AC injections, mice were immunized SC with B6 spleen cells seven days later, and DTH responses were evaluated seven days after the SC immunization. DTH responses in mice with allergic conjunctivitis were the same as those in the PBS control mice, indicating that Th2-based inflammation did not affect the induction of ACAID (Figure 16). Both the allergic conjunctivitis group and the PBS controls had a DTH response that was significantly lower than the positive SC controls.

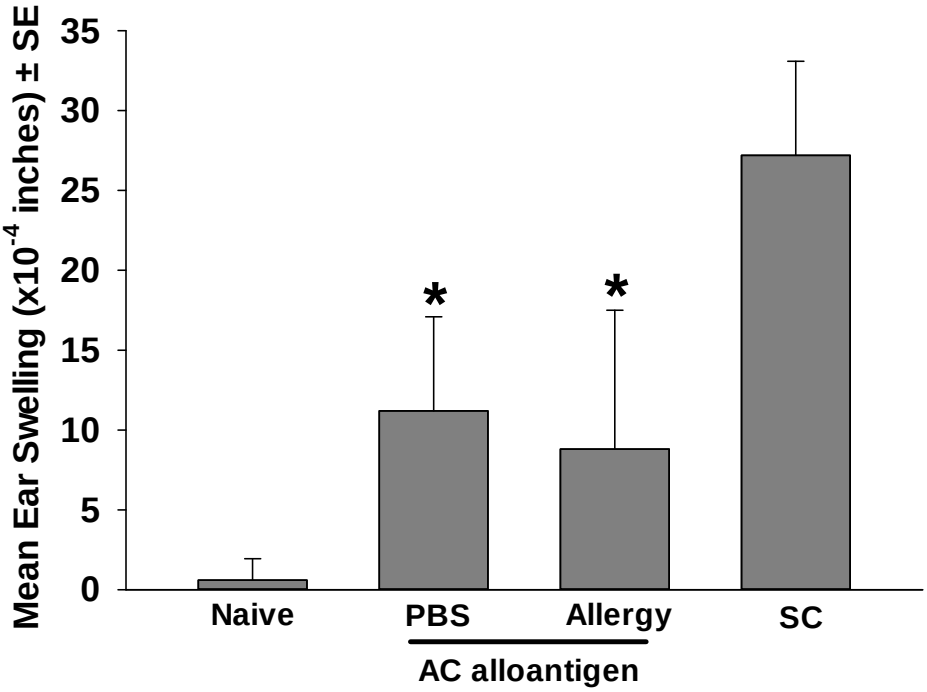


Figure 16. Effect of allergic conjunctivitis on ACAID. Allergic conjunctivitis was induced by immunization followed by topical challenge with SRW pollen prior to AC injection with B6 alloantigens. All groups were SC immunized with B6 spleen cells seven days before DTH was assessed. The positive control group was immunized SC but was not primed in the eye. Graph is representative of two independent experiments (n=5 mice/group/experiment); * $p < 0.05$

Allergic conjunctivitis does not lead to the generation of donor-specific CTL

Patients undergoing a corneal transplant for correction of keratoconus, a condition in which the cornea is cone-shaped but remains avascular, have an acceptance rate of 90% [177]. In patients considered to be high-risk due to pre-existing corneal vascularization, the survival rate drops to about 35% [194]. Murine models of corneal transplantation have confirmed the exacerbation of rejection in hosts deemed high-risk due to the presence of a vascularized graft bed [143]. This increase in graft rejection was correlated with the development of donor-specific $CD8^+$ CTL that were not seen in normal mice that had rejected their corneal allografts (non-allergic rejectors) [24].

In our next aim, we wanted to determine if allergic conjunctivitis caused a qualitative difference in the alloimmune response as was seen in other high-risk hosts. Therefore, it was hypothesized that mice with allergic conjunctivitis that had rejected their corneal allografts (allergic rejectors), generated donor-specific CTL that were responsible for the increased rejection. To test this, splenocytes from allergic rejectors were isolated one to seven days after rejection and were stimulated directly using B6 donor APC or indirectly using recipient BALB/c APC pulsed with donor B6 antigens and assessed for donor-specific CTL responses. Like non-allergic rejectors, allergic rejectors did not generate CTL to donor alloantigens (Figure 18A and 18B). To confirm the presence of CTL, in addition to a SC positive control, mice with pre-vascularized corneas were generated by placing sutures in the corneas two weeks prior to receiving corneal allografts. As expected, 100% of these high-risk hosts underwent rejection after which their splenocytes were harvested and tested for CTL activity using both direct and indirect stimulation. Although there was killing seen in the positive control group, no killing was seen in the group with pre-vascularized graft beds (Figure 17A and 17B). It was possible that

CTL were confined to the draining lymph nodes rather than the spleen. Accordingly, the cervical lymph nodes were harvested and stimulated directly using B6 APC; however no CTL activity was detected in the high-risk group with pre-vascularized graft beds that had rejected 100% of their corneal allografts (Figure 17C). All together, these results suggested that allergic conjunctivitis does not cause a qualitative difference in the alloimmune response.

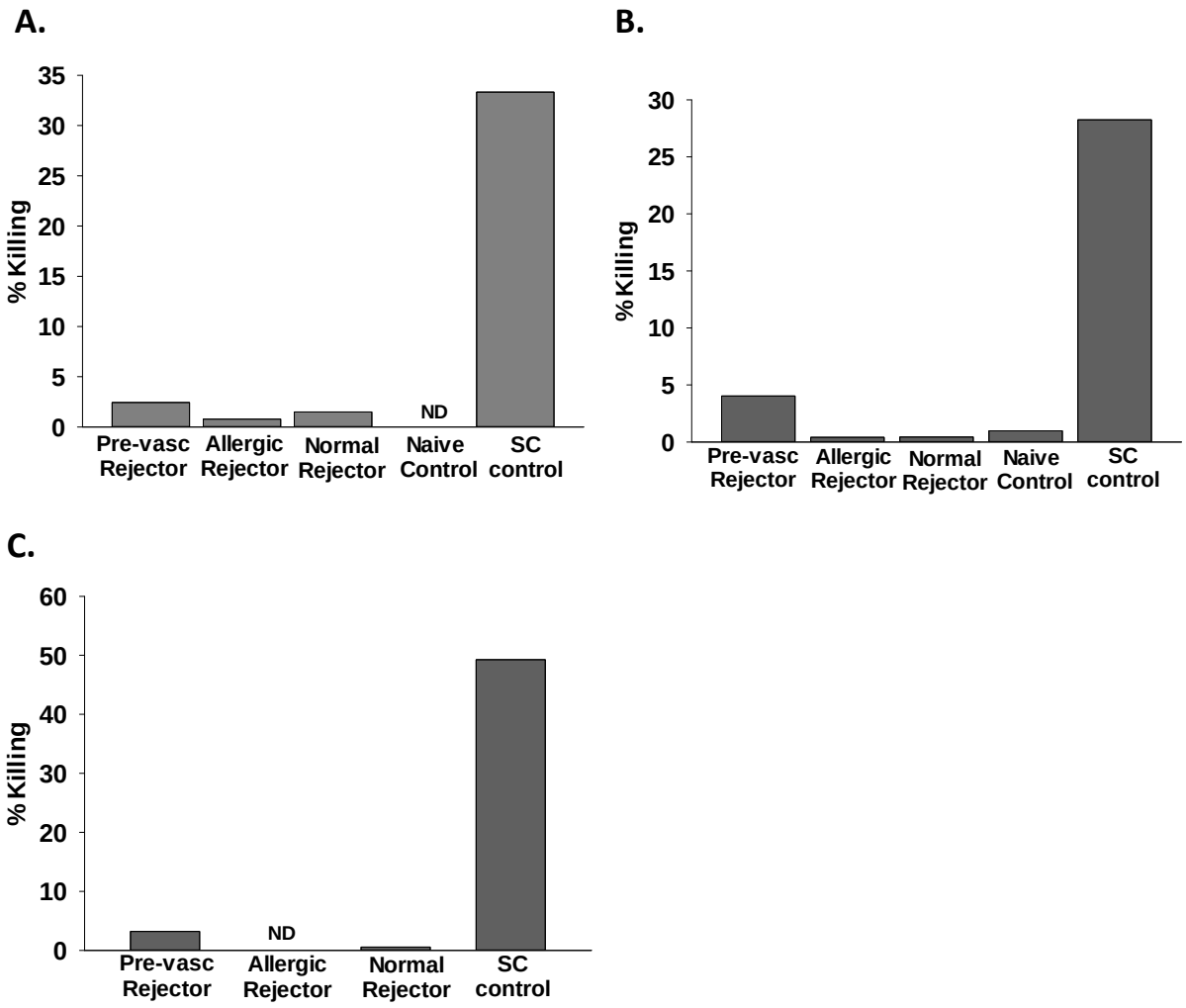


Figure 17. CTL responses of grafted mice with allergic conjunctivitis to donor B6 antigen. BALB/c mice with allergic conjunctivitis were grafted with B6 corneas 17 days after initial sensitization. One to seven days after corneal allograft rejection, splenocytes from allergic, non-allergic, or pre-vascularized rejectors were stimulated A) directly (with mitomycin-C-treated B6 APC) or B) indirectly (BALB/c APC pulsed with B6 antigen) for 96 hr. C) Cells from LN were stimulated directly using donor B6 APC. Responder cells were used in a CTL assay against B16LS9HG target cells. Graph is representative of four independent experiments (n=3 mice/group/experiment).

Lymphocytes from allergic rejectors do not have enhanced proliferation in response to donor alloantigens compared to non-allergic rejectors

We next wanted to determine if allergic conjunctivitis caused a quantitative difference in the alloimmune response. More specifically, experiments were performed to determine if $CD4^+$ T cells from allergic rejectors mounted a more robust response to donor alloantigen compared to non-allergic rejectors. First, the proliferative response of BALB/c $CD4^+$ T cells from mice that had been previously sensitized and challenged with SRW pollen (mice that developed allergic conjunctivitis) without a corneal allograft was compared to naïve (non-allergic) mice. In the primary mixed lymphocyte reaction (MLR), results showed that lymphocytes from mice with allergic conjunctivitis and naïve mice had comparable proliferative responses to B6 alloantigen (Figure 18). Next, the response of $CD4^+$ T cells from allergic rejectors and non-allergic rejectors to donor B6 alloantigen was assessed. In the secondary MLR, results showed that $CD4^+$ T cells from allergic rejectors and non-allergic rejectors also had comparable proliferation to B6 alloantigen (Figure 19). These results indicate that the increase in corneal allograft rejection in mice with allergic conjunctivitis was not due to an enhanced lymphoproliferative response against donor alloantigens.

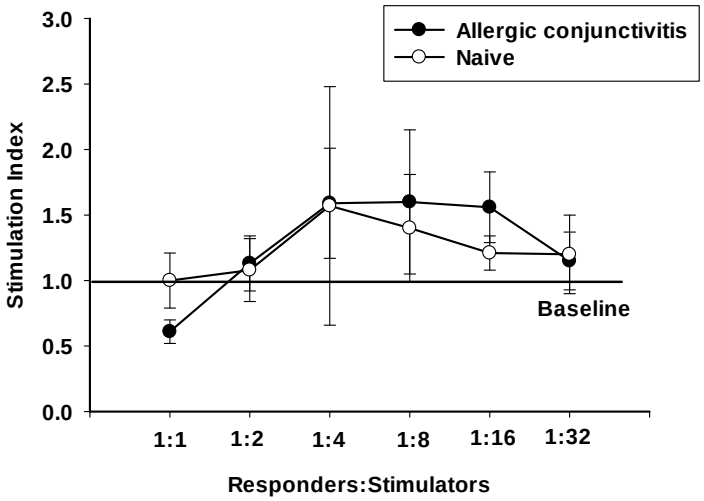


Figure 18. Proliferative responses of $CD4^+$ T cells from naive mice and mice with allergic conjunctivitis to donor B6 antigen. Allergic conjunctivitis was induced in BALB/c mice and following seven day challenge with SRW pollen, $CD4^+$ T cells from allergic (or naïve) mice were stimulated indirectly with BALB/c APC pulsed with B6 antigen. Lymphocyte proliferation was determined by ^{3}H-thymidine incorporation 72 hr later. This graph is representative of three independent experiments (n=5 mice/group/experiment); * $p < 0.05$

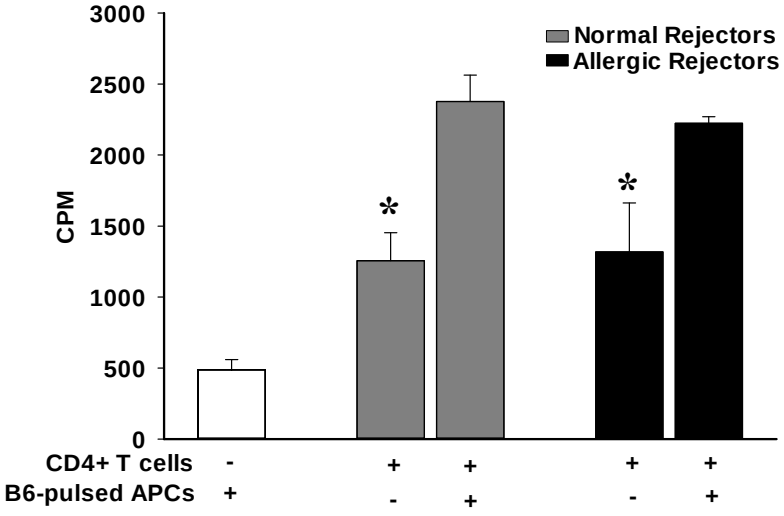


Figure 19. Proliferative responses of CD4$^+$ T cells from non-allergic and allergic grafted mice to donor B6 antigen. BALB/c mice with or without allergic conjunctivitis were grafted with B6 corneal allografts. One to seven days after rejection, CD4$^+$ T cells were isolated and stimulated indirectly with BALB/c APC pulsed with B6 alloantigens. Lymphocyte proliferation was determined by ^{3}H-thymidine incorporation 96 hr later. This graph is representative of two independent experiments (n=5 mice/group/experiment); * $p < 0.05$

IL-4 inhibits Treg suppression of effector T cells in vitro

The results thus far have indicated that allergic conjunctivitis did not cause a qualitative or quantitative difference in the alloimmune response that would explain the increase in corneal allograft rejection. Several investigators have established the importance of $CD4^+CD25^+$ Tregs in corneal transplantation [171, 172]. Depleting Tregs with anti-CD25 antibody treatment resulted in an increase in corneal allograft rejection from 50% to 100% [171]. Moreover, adoptive transfer of $CD4^+CD25^+$ T cells into naïve BALB/c allograft recipients prior to placing a B6 corneal allograft resulted in an increase in corneal allograft survival [172].

Therefore, the next aim was to determine if allergic conjunctivitis affected the Tregs that support corneal allograft survival. One study suggested this possibility when the function of nTregs was examined in allergic AHR and showed that in the presence of IL-4, the suppressive function of nTregs was inhibited [195]. To assess if the Th2 cytokines, IL-4, IL-5, and IL-13, affected the suppressive function of Tregs induced after corneal transplantation, an *in vitro* suppression assay was performed. $CD4^+CD25^+$ putative Tregs were isolated from BALB/c mice that had accepted their B6 corneal allografts (graft acceptors) and were co-cultured with anti-CD3-stimluated $CD4^+$ effector T cells isolated from naïve animals in the presence or absence of various Th2 cytokines. Lymphocyte proliferation was subsequently measured by thymidine incorporation. Tregs from graft acceptors suppressed the proliferation of stimulated $CD4^+$ effector T cells, which agreed with the results from other investigators [169, 172]. This suppression was abolished in the presence of IL-4, but was unaffected in the presence of IL-5 or IL-13 (Figure 20).

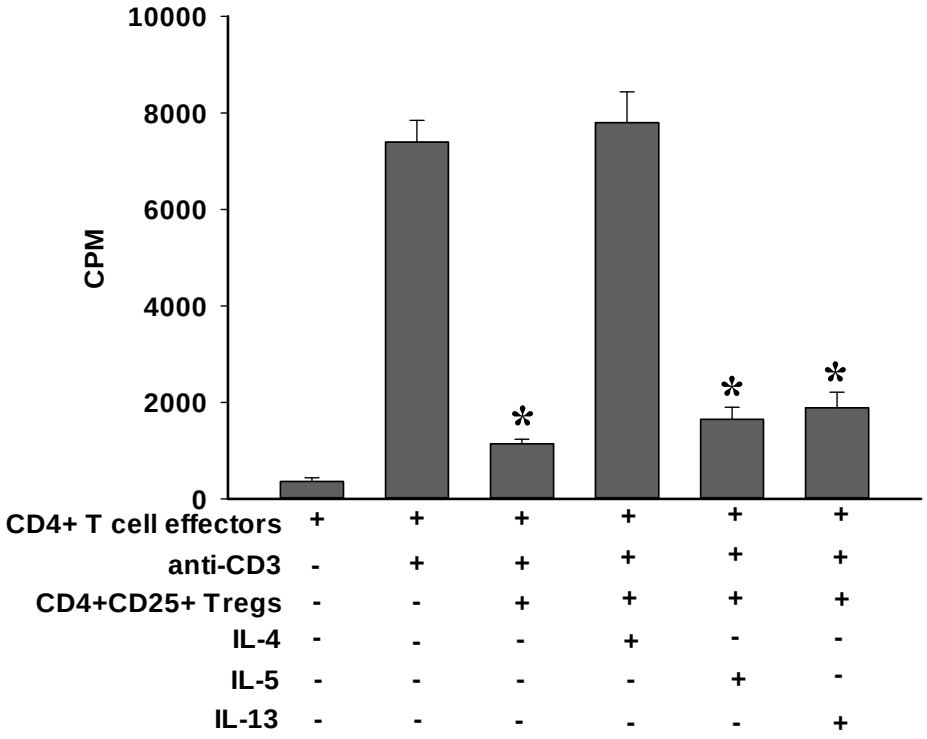


Figure 20. Suppressive ability of Tregs in the presence of Th2 cytokines. The ability of $CD4^+CD25^+$ T cells (Tregs) isolated from corneal allograft acceptors to suppress naïve anti-CD3-stimulated $CD4^+$ T cells was examined in the presence or absence of Th2 cytokines (IL-4, IL-5, or IL-13). Proliferation was determined by ^{3}H-thymidine incorporation 96 hr later. Graph is representative of four independent experiments (n=3 mice/group/experiment); * $p < 0.05$

Preventing Tregs from proliferating does not affect their suppressive function

Tregs have been characterized as having anergic properties [195, 196]. It was important to establish that the proliferation seen in our suppression assays was due to proliferation of the effector T cell population and not from the Tregs. Tregs from graft acceptors were treated with mitomycin-C to prevent possible Treg proliferation. These treated Tregs were then used in a suppression assay as previously described in the presence or absence of IL-4. Results showed that even when Treg proliferation was inhibited, Tregs were capable of suppressing effector T cell proliferation (Figure 21). As seen Figure 20, this suppression was inhibited by the presence of IL-4 (Figure 21).

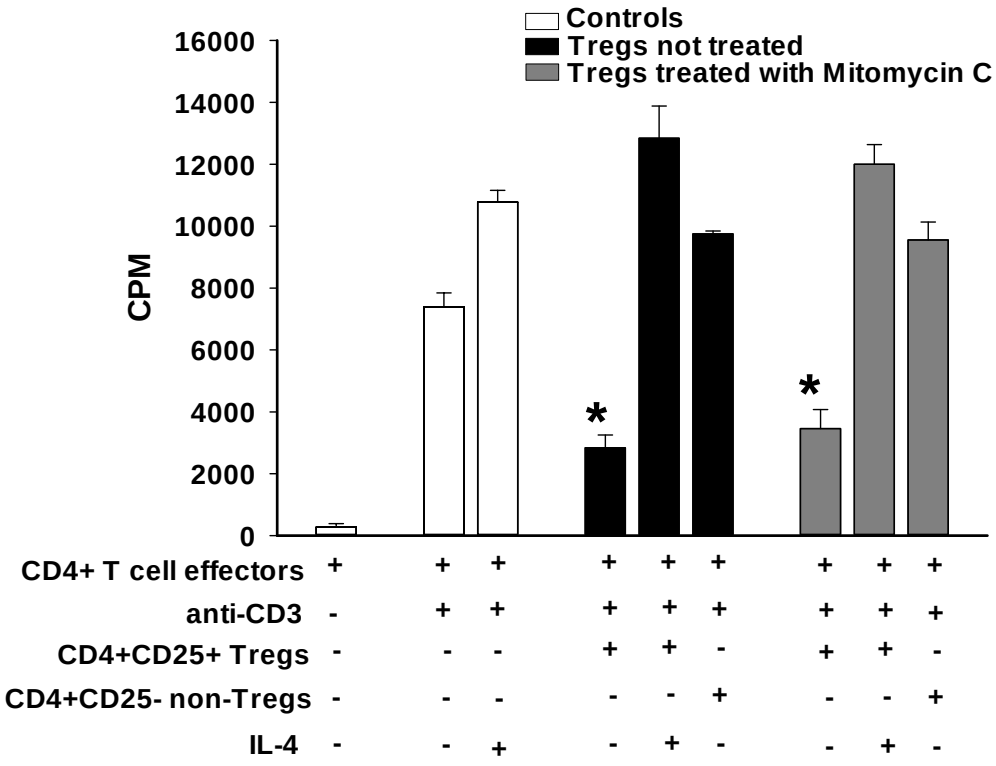


Figure 21. Mitomycin-C treatment does not affect Treg function. $CD4^+CD25^+$ Tregs were isolated from corneal allograft acceptors and co-cultured with anti-CD3-stimulated $CD4^+$ T cells from naïve WT BALB/c mice. These same Tregs were treated with mitomycin-C for 30 min before co-culturing with $CD4^+$ T cells. Proliferation was determined by ^{3}H-thymidine incorporation 96 h later. Graph is representative of three independent experiments (n=4 mice/group/experiment); * $p < 0.05$

IL-4 inhibits Treg suppression of effector T cells in vivo

To confirm that IL-4 affected Treg suppressive activity, a previously described local adoptive transfer (LAT) assay was performed [169]. In this *in vivo* suppression assay, Tregs from graft acceptors were co-injected with effector T cells from non-allergic rejectors and APC that had been pulsed with B6 alloantigens into the ears of naïve mice in the presence or absence of IL-4. Twenty-four hours later DTH responses were measured. The group that received Tregs had a reduction in ear swelling compared to mice that only received effector T cells and APC, which indicated that co-injected Tregs suppressed DTH responses (Figure 22A). The reduction in ear swelling was lost in the group in which IL-4 was added to the co-injected cells. Injection of Tregs, effector T cells, and IL-5 or IL-13, demonstrated a reduction in ear swelling (Figure 22A), although it was not as reduced as having Tregs with no additional cytokines. It is important to note that DTH responses occur as a result of T cells proliferation in addition to the secretion of cytokines that cause vessel dilation and the recruitment of other inflammatory cells, such as macrophages, that lead to the accumulation of plasma and tissue damage. Therefore, IL-5 or IL-13 may be enhancing factors that mediate DTH responses and Tregs cannot mediate suppression to the extent where no exogenous cytokine are added.

The results from the LAT assay raised concern that IL-4 alone could cause an inflammatory response. To test this hypothesis, a LAT assay was performed with IL-4 and APC that had been pulsed with B6 alloantigens. If IL-4 caused an inflammatory response, then this group would have ear swelling that was comparable to the positive control. As seen in Figure 22B, IL-4 added to APC did not cause an increase in ear swelling, indicating that IL-4 alone was not pro-inflammatory. Together, these results suggested that during allergic inflammation, IL-4,

but not IL-5 or IL-13, was responsible for blocking Treg inhibition of effector T cell proliferation.

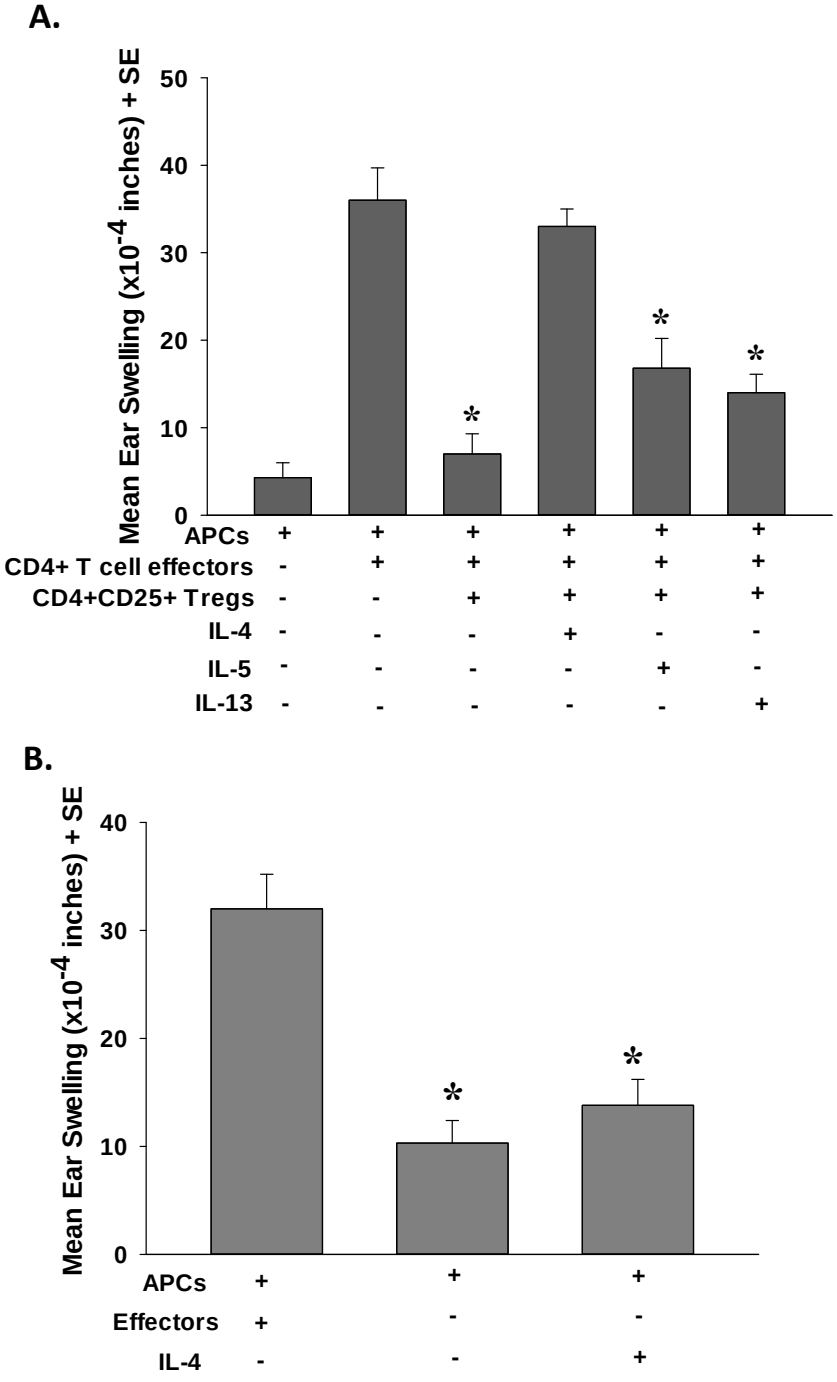


Figure 22. IL-4 inhibits Treg suppression *in vivo*. A) $CD4^+CD25^+$ Tregs were isolated from corneal allograft acceptors and mixed with $CD4^+$ T cells from corneal allograft rejectors and APC pulsed with B6 antigen in the presence or absence of IL-4. Cells were used in a LAT assay for DTH ear swelling assay. B) APCs pulsed with B6 antigen in the presence or absence of IL-4 were used in a LAT assay for DTH ear swelling. Graph is representative of two independent experiments (n=5 mice/group/experiment); * $p < 0.05$

IL-4 does not affect the development of Tregs induced by corneal transplantation

Based on the results from the *in vitro* suppression assay, it was unclear whether IL-4 affected the Tregs or the effector T cells, or both. Allergic conjunctivitis, and more specifically IL-4, could have been affecting Treg development, and in turn, rendering Tregs incapable of suppressing effector T cells. It is also possible that IL-4 caused effector T cells to become resistant to Treg suppression. To first determine if IL-4 affected Treg function, the suppressive ability of Tregs isolated from mice with allergic conjunctivitis that had a B6 corneal allograft (allergic grafted mice) was assessed. As noted before, under non-allergic conditions, only 50% of BALB/c mice that received a B6 corneal allograft had Treg activity [197]. If IL-4 affected Tregs, rather than observing Treg activity in 50% of the recipient mice, zero activity would have been seen. It is also important to note that prior to placing a B6 allograft, there is no method to predict which 50% of BALB/c recipients will develop Tregs. Therefore, putative Tregs were isolated from individual allergic grafted mice and co-cultured with anti-CD3-stimulated effector T cells isolated from naïve mice. A representative figure from nine allergic grafted mice showed that there was Treg activity in 50% of the mice as seen by the suppression of proliferation of $CD4^+$ T cells (Figure 23A). For comparison, Tregs from individual non-allergic mice that had a B6 corneal allograft (non-allergic grafted mice) were used. The results from the suppressive profile of Tregs from both non-allergic and allergic grafted mice revealed no differences (Figure 23B). These results suggested that allergic conjunctivitis does not affect the generation of Tregs, and these Tregs have suppressive functions when removed from an IL-4-rich environment.

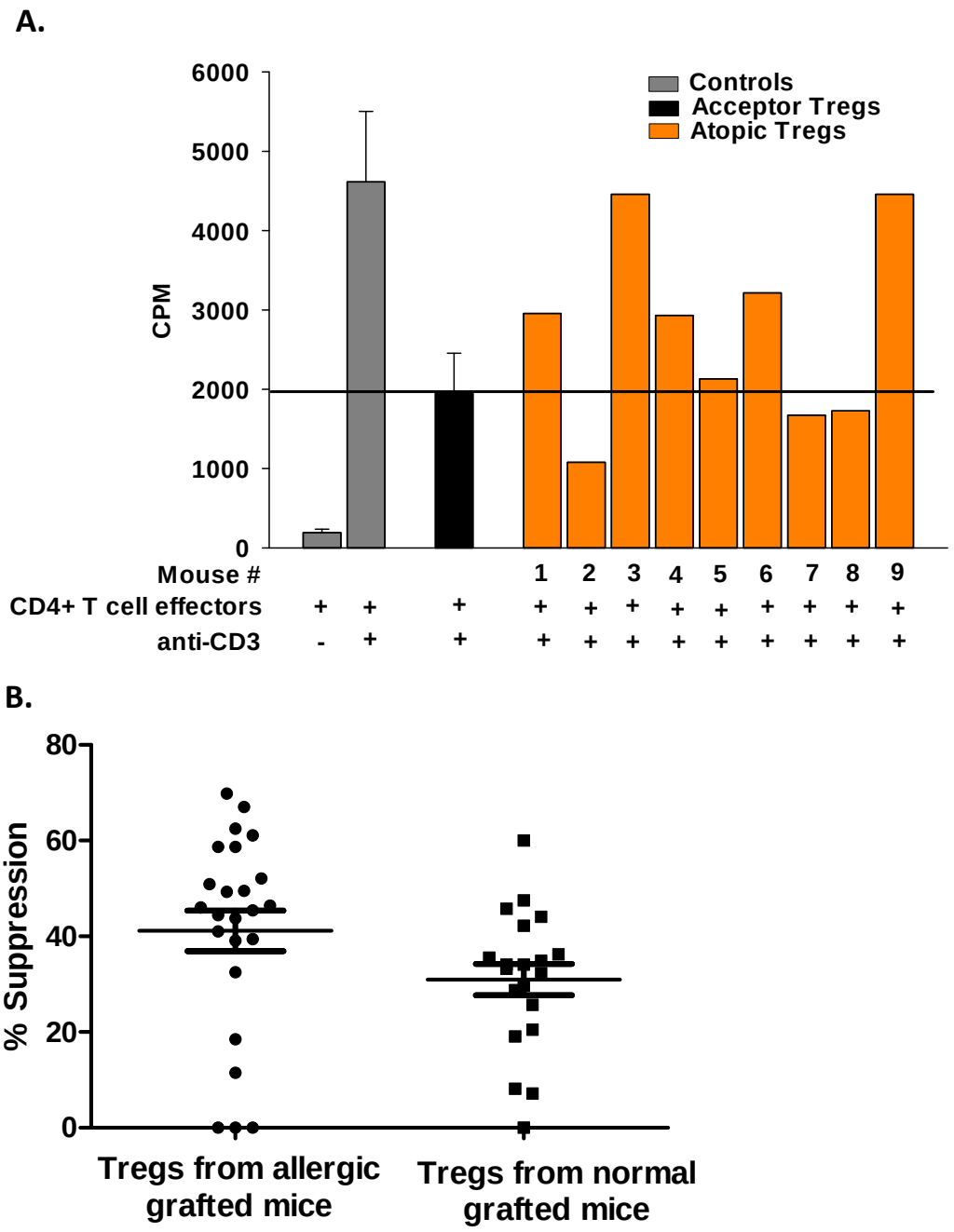


Figure 23. Suppressive profile of Tregs from allergic and non-allergic grafted mice. $CD4^+CD25^+$ Tregs were isolated from individual non-allergic and allergic grafted mice. Tregs were co-cultured with anti-CD3-stimulted $CD4^+$ T cells from naïve WT BALB/c mice. Proliferation was determined by ^{3}H-thymidine incorporation 96 h later. A) Suppression by Tregs from allergic rejectors. Graph is representative of three independent experiments (n=9 mice/group/experiment). B) Suppressive profile of Tregs from allergic and non-allergic grafted mice. Graph shows data from three independent experiments from the allergic group (n=25 total) and two independent experiments from the non-allergic group (n=20 total).

IL-4 renders effector T cells resistant to Treg suppression

Since Tregs isolated from mice with allergic conjunctivitis displayed suppressive ability comparable to Tregs from non-allergic mice, we hypothesized that IL-4 rendered effector T cells resistant to Treg suppression. To test this, $CD4^+$ T cells from IL-4R$\alpha^{-/-}$ mice were utilized. Tregs from graft acceptors were co-cultured with IL-4R$\alpha^{-/-}$ effector T cells in the presence or absence of IL-4. As seen in Figure 24, Tregs suppressed the proliferation of anti-CD3-stimulated IL-4R$\alpha^{-/-}$ effector T cells in the absence of IL-4. Interestingly, suppression of IL-4R$\alpha^{-/-}$ effector T cells was unaffected when IL-4 was present in the cultures. Together, these results suggested that IL-4 renders effector T cells resistant to Treg suppression, and therefore, hosts with allergic diseases experience an increase in corneal allograft rejection.

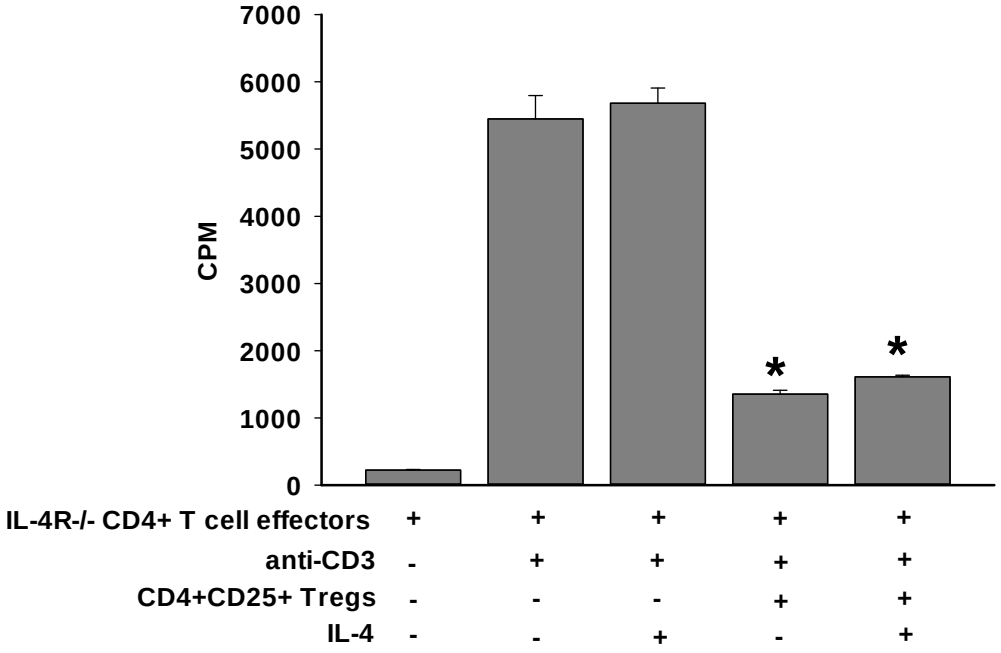


Figure 24. Suppression assay using effector T cells that cannot respond to IL-4. $CD4^+CD25^+$ Tregs were isolated from corneal allograft acceptors and co-cultured with anti-CD3-stimulated $CD4^+$ T cells from naïve IL-4R$\alpha^{-/-}$ BALB/c mice in the presence or absence of IL-4. Proliferation was determined by ^{3}H-thymidine incorporation 96 hr later. Graph is representative of three independent experiments (n=4 mice/group/experiment); * $p < 0.05$

IL-4Rα$^{-/-}$ mice develop allergic conjunctivitis

Since IL-4 rendered effector T cells resistant to Treg suppression, we hypothesized that IL-4Rα$^{-/-}$ mice with allergic conjunctivitis would experience the same 50% corneal allograft survival seen in normal, non-allergic controls. In order to test this hypothesis, the degree to which IL-4Rα$^{-/-}$ mice could develop allergic conjunctivitis first needed to be determined. Allergic conjunctivitis was induced as previously described and both the clinical phenotype and the eosinophilic infiltration into the conjunctiva was assessed. IL-4Rα$^{-/-}$ mice developed the clinical phenotype of allergic conjunctivitis that was comparable to WT mice (Figure 25A). There was also no difference in the number of eosinophils in the conjunctivas of IL-4Rα$^{-/-}$ mice compared to WT mice (Figure 25B). In addition, IL-4Rα$^{-/-}$ mice with allergic conjunctivitis secreted IL-4, IL-5, IL-13, and IFN-γ, to the same degree as WT mice (Figure 26). The results demonstrated that IL-4Rα$^{-/-}$ mice developed allergic conjunctivitis to the same degree as WT mice.

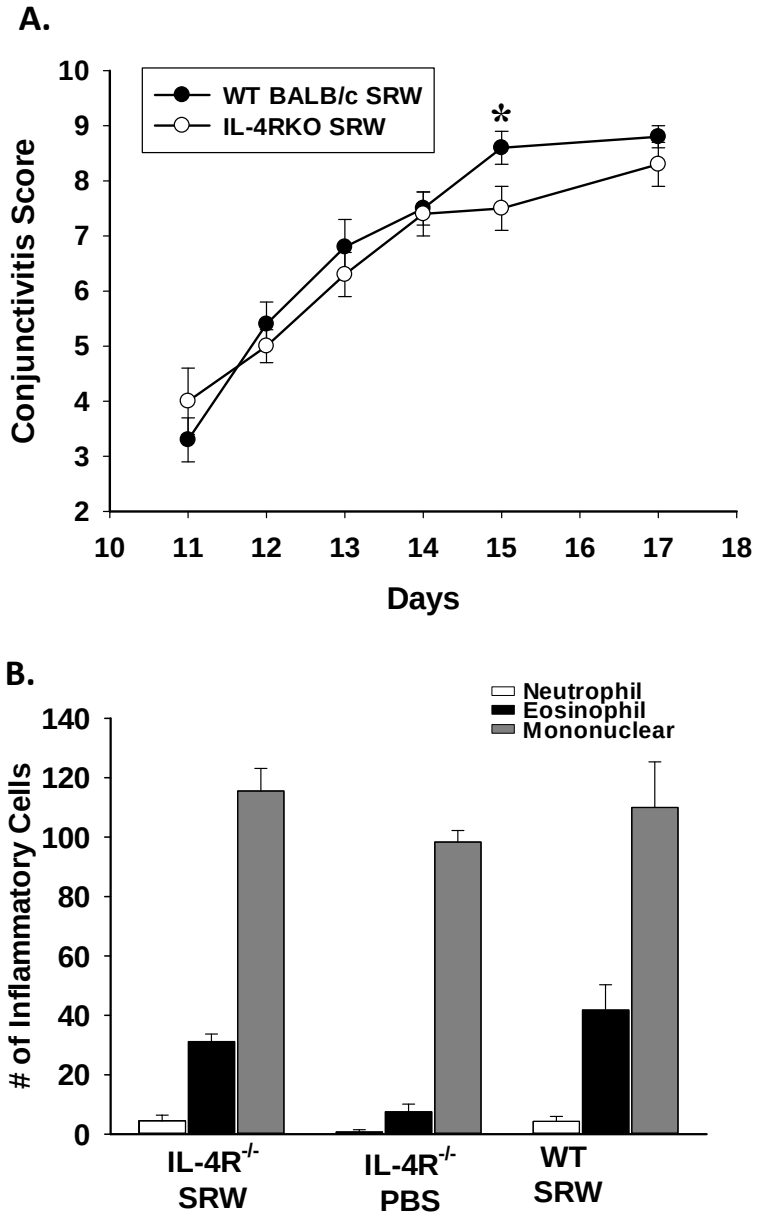


Figure 25. Allergic conjunctivitis in IL-4Rα$^{-/-}$ mice. A) Clinical allergic conjunctivitis scores in WT BALB/c and IL-4R$^{-/-}$ mice sensitized and challenged with SRW pollen. Graph shows the average of two independent experiments (n=10 total). B) Eosinophilic infiltrations into the conjunctivae of SRW pollen-challenged mice. Graph is representative of two independent experiments (n=5 mice/group/experiment). * $p<0.05$

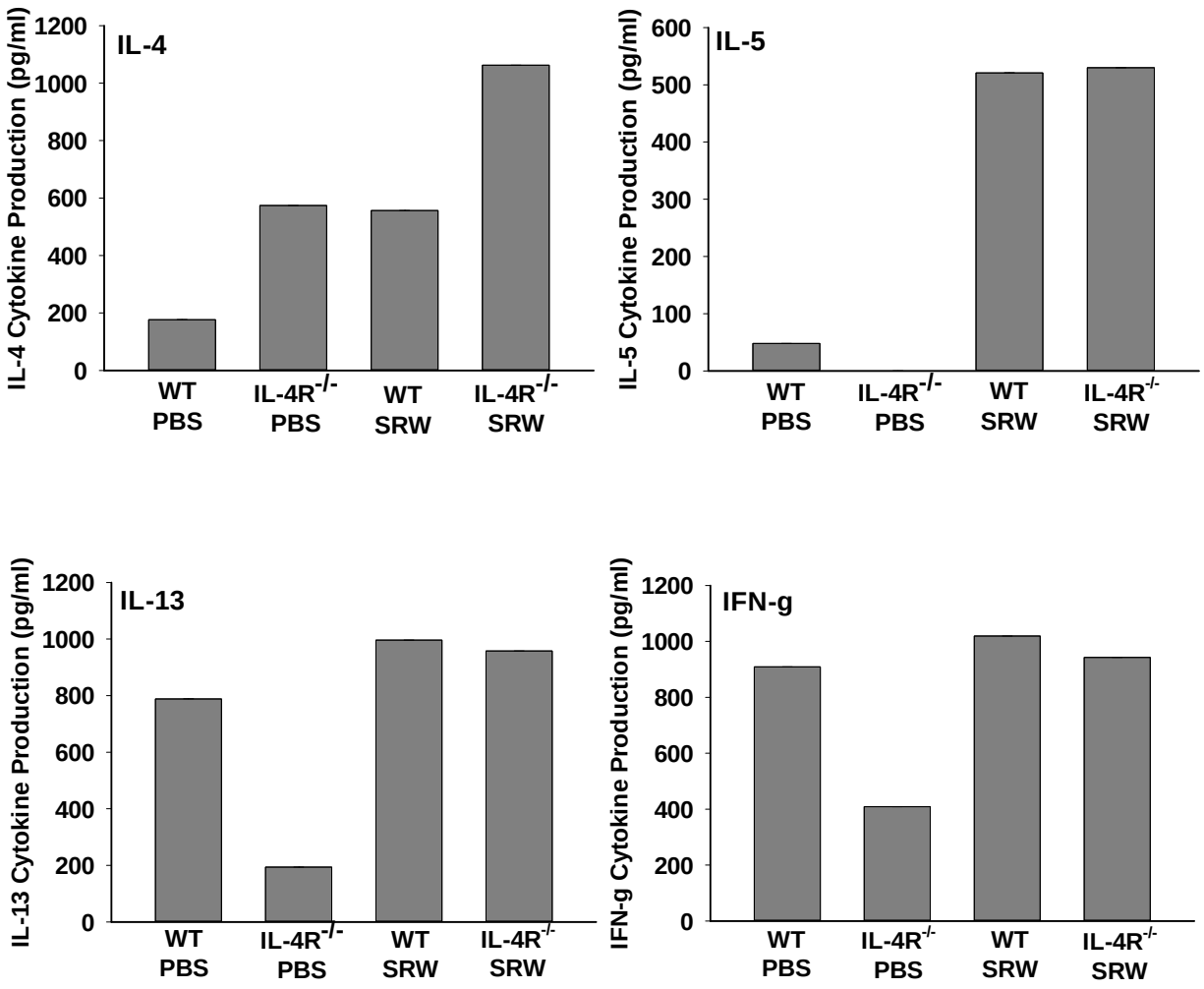


Figure 26. IL-4Rα$^{-/-}$ mice secrete normal levels of Th2 cytokines. Bulk splenocytes were incubated with SRW extract. Supernatants were collected 48 hr after *in vitro* culture and analyzed by ELISA. This graph is representative of two independent experiments (n=5 mice/group/experiment). * $p < 0.05$

Allergic BALB/c IL-4Rα$^{-/-}$ mice with B6 corneal allografts have the same incidence of rejection as non-allergic WT mice

Since it was established that IL-4Rα$^{-/-}$ mice developed allergic conjunctivitis to the same degree as WT mice, graft survival was assessed to determine if the inability to respond to IL-4 affected graft survival. Accordingly, allergic conjunctivitis was induced in IL-4Rα$^{-/-}$ mice prior to placing a B6 corneal allograft. B6 grafted non-allergic IL-4Rα$^{-/-}$ mice had the same 50% graft survival as normal, non-allergic controls (Figure 27). Interestingly, IL-4Rα$^{-/-}$ mice with allergic conjunctivitis that also received a corneal allograft had a 50% graft survival similar to non-allergic controls (Figure 27). These results suggested that allergic conjunctivitis does not exacerbate corneal allograft rejection unless host effector T cells are able to respond to IL-4.

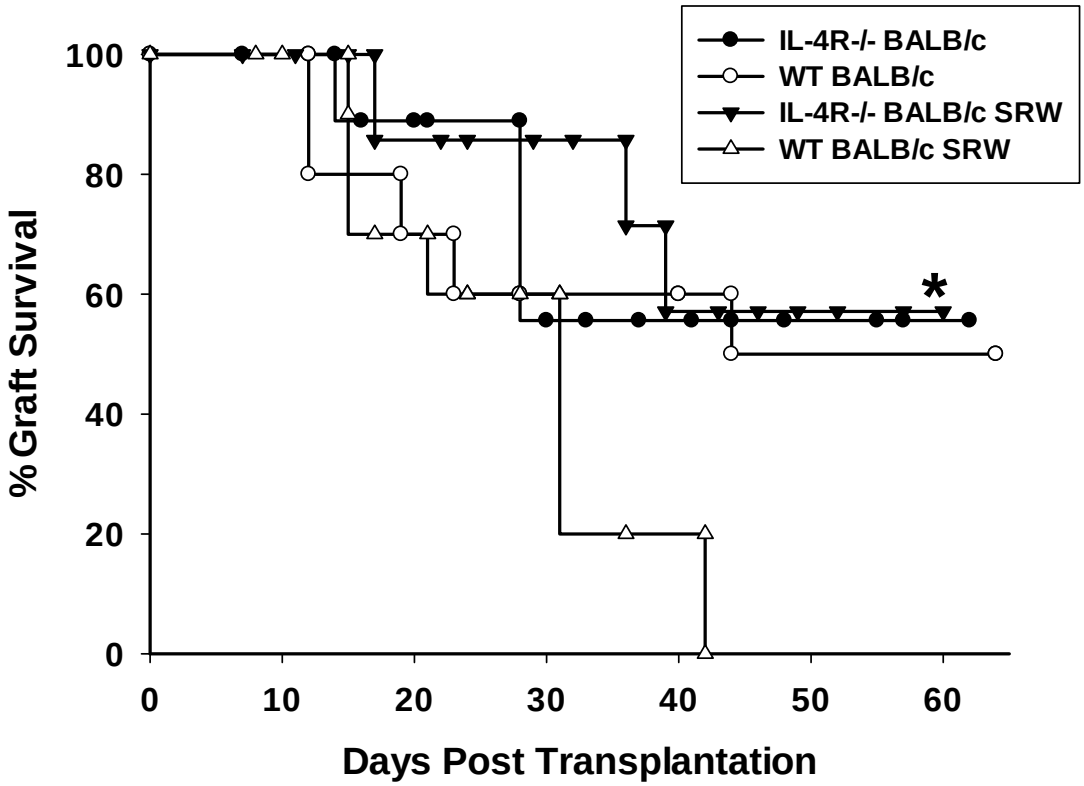


Figure 27. Allograft survival is unimpaired in IL-4Rα$^{-/-}$ BALB/c grafted mice with allergic conjunctivitis. Allergic conjunctivitis was induced in IL-4Rα$^{-/-}$ (▼) or WT BALB/c (Δ) mice prior to receiving B6 corneal allografts. Naïve IL-4Rα$^{-/-}$ (●) or WT BALB/c (○) mice were grafted with B6 corneal allografts. Graph is representative of two independent experiments (n=10 mice/group/experiment); * $p<0.05$

Neutralizing IL-4 in WT mice with allergic conjunctivitis restores corneal allograft survival

The previous results indicated that effector T cells that are unresponsive to IL-4 become amenable to Treg suppression even in hosts with allergic conjunctivitis. This observation gave insight to the kinds of treatments that might benefit patients with pre-existing allergic diseases and are destined to receive a corneal transplant. We wanted to determine if neutralizing IL-4 would give the same results found in IL-4Rα$^{-/-}$ mice with allergic conjunctivitis. This could be a potential therapy for patients suffering from allergic diseases at the time they are undergoing a corneal transplant. To recapitulate what occurred in a clinical setting, WT mice were sensitized and challenged with SRW pollen to induce allergic conjunctivitis. On the same day that the allergic mice received a B6 corneal transplant anti-IL-4 antibody treatment was initiated. As seen in Figure 28, anti-IL-4 treatment in mice with allergic conjunctivitis and that received corneal allografts resulted in 50% graft survival, indicating that neutralizing IL-4 *in vivo* renders effector T cells amenable to Treg suppression even in the presence of continuous exposure to the allergen.

We also wanted to determine if the 50% incidence of corneal allograft rejection in non-allergic BALB/c mice was due to these mice constitutively producing IL-4. Accordingly, non-allergic recipients were treated with anti-IL-4 antibody at the time of transplantation. The results showed that anti-IL-4 treatment did not increase corneal allograft survival above the normal 50% (Figure 28).

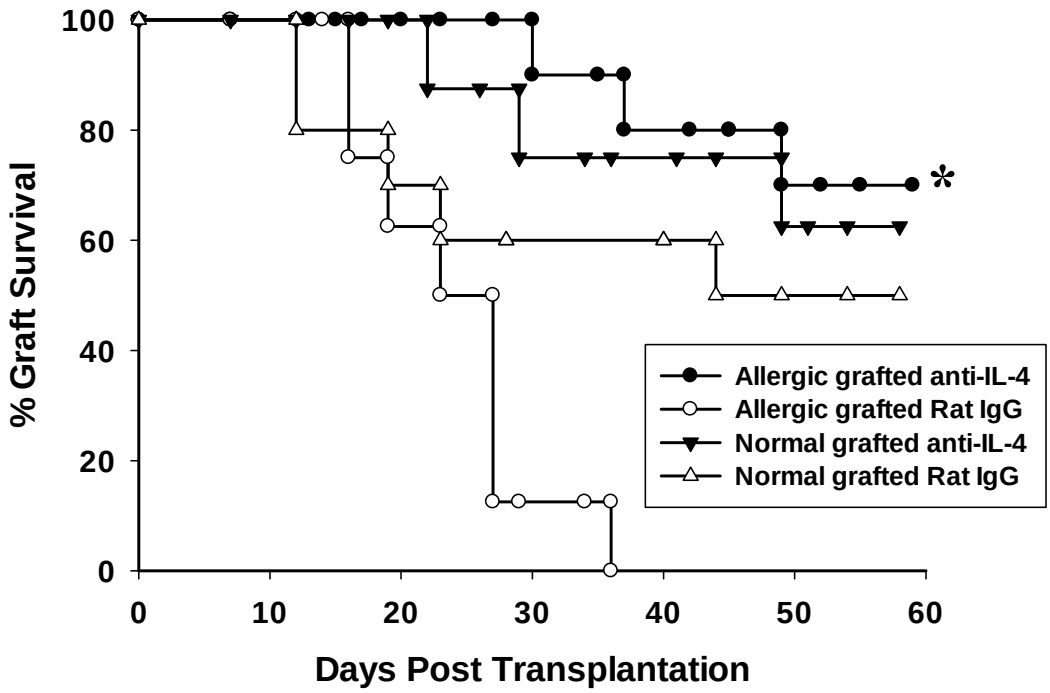


Figure 28. Anti-IL-4 treatment restores corneal immune privilege in mice with allergic conjunctivitis. Allergic conjunctivitis was induced in WT BALB/c mice. Anti-IL-4 (●) or isotype control antibody (○) treatment was initiated on the day mice received corneal allografts. Non-allergic mice were also treated with anti-IL-4 (▼) or isotype control (Δ). Graph is representative of two independent experiments (n=10 mice/group/experiment); * $p < 0.05$

Resistance to Treg suppression is transient

The results to this point established that IL-4 renders effector T cells resistant to Treg suppression; however, it is unclear whether or not this resistance is permanent. To assess this, allergic conjunctivitis was induced in WT BALB/c mice as previously described. The day these allergic mice received a B6 corneal allograft, SRW pollen challenge was stopped for a period of 14 or 28 days. After this rest period, SRW pollen challenge was resumed for the remainder of the experiment. Mice that were isolated for 28 days from their allergen had the same 50% survival as non-allergic WT mice (Figure 29). Interestingly, resting for 14 days also restored 50% graft survival (Figure 29). These results suggested that effector T cell resistance to Treg suppression is transient and can be restored when the hosts are isolated from their allergen for a period as short as 14 days.

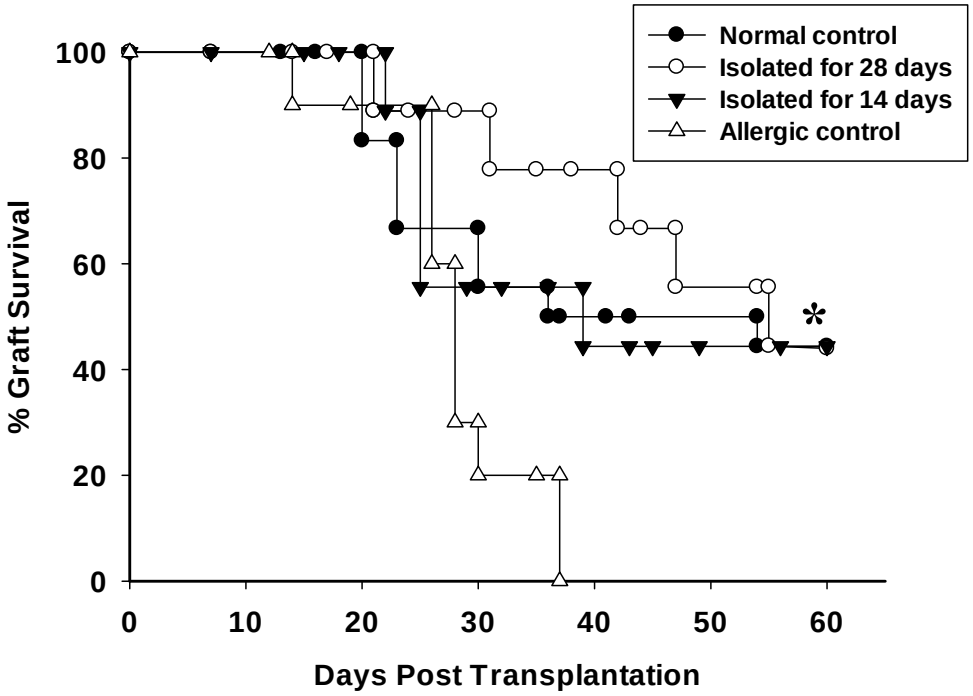


Figure 29. Isolation from SRW pollen restores corneal immune privilege. Allergic conjunctivitis was induced in WT BALB/c mice. The day mice underwent a corneal allograft, mice were isolated from SRW pollen for 28 days (○) or 14 days (▼). Non-allergic controls (●) were not sensitized or challenged with pollen, while allergic controls (Δ) were not isolated and had continuous challenge with SRW pollen. Graph is representative of two independent experiments (n=10 mice/group/experiment); * $p < 0.05$

CHAPTER FOUR

DISCUSSION

Clinical relevance of this study

Corneal transplantation is arguably the most successful form of tissue transplantation. Transplants are routinely performed without HLA matching and without the use of systemically administered immunosuppressive drugs. Ocular immune privilege has been recognized for over 50 years and is a result of three processes: 1) preventing the induction of alloimmune responses, 2) deviation of the immune response to a tolerogenic response, and 3) the presence of factors that block the function of immune effector elements at the graft/host interface [25]. Immune rejection remains the leading cause of corneal allograft rejection. According to the National Eye Institute (NEI), about 40,000 corneal transplants are performed each year with about 20% of patients (between 6,000 and 8,000) experiencing rejection. It is estimated that long-term survival at 10 years is 60% [75]. High-risk patients, those that have pre-existing diseases or vascularization to the cornea, have a much lower success rate [74, 75].

There is some debate among physicians regarding allergic diseases and their effect on corneal transplantation. Some groups have found no difference in the prevalence of graft rejection between patients with a history of allergies compared to controls [198]. Others have observed that having severe ocular allergies elevates the risk of graft rejection [95-97]. With the cost of corneal transplantation at approximately $20,000, finding better treatments for high-risk patients to increase their graft survival is needed [199]. This study provides new insights into how allergic diseases affect corneal allograft survival and sheds light as to the types of therapies needed for increasing graft survival in patients with allergic diseases such as allergic conjunctivitis.

ALLERGIC CONJUNCTIVITIS

Allergic conjunctivitis is an immediate hypersensitivity reaction mediated by a Th2-immune response and the preferential generation of IgE antibodies. In the early phase reaction, the allergen cross-links IgE antibodies bound via FcεRI on the surface of mast cells [100]. This event occurs within minutes of allergen exposure and triggers the degranulation of mast cells releasing mediators such as histamine. The late phase reaction is evident 6-12 hours after allergen challenge and is characterized by an inflammatory infiltrate consisting of neutrophils and a preponderance of eosinophils [100]. Th2 cells and their cytokines are associated with many allergic disorders such as atopic dermatitis and asthma [200]. Although antigen-specific Th2 cells are considered key in the pathogenesis of allergic diseases [107], recent findings indicate that innate T cells such as NKT and γδ T cells also have an important role in allergic diseases [106, 137, 201]

Innate T cells in Allergic Conjunctivitis

In this study, we wanted to establish that the mouse model of SRW pollen-induced allergic conjunctivitis mimicked what was seen in a clinical setting. By assessing the development and expression of allergic conjunctivitis in different hosts, we found that both NKT and γδ T cells are required for maximal expression.

NKT cells

NKT cells are unique subgroup of lymphocytes that differ from conventional αβ T cells in that they recognize lipid, and not peptide, antigens and upon stimulation, produce both Th1 and Th2 cytokines [121, 122]. Two different subsets of NKT cells have been described: type I NKT cells that express invariant Vα14Jα18 TCR in the mouse or Vα24Jα18 in humans, and type II NKT cells that express more diverse TCRs [123]. Type I NKT cells recognize lipid antigens,

such as α-galactosylceramide (α-GalCer), presented by a nonclassical MHC molecule, CD1d [123]. In tumor immunity, type I NKT cells have been shown to primarily mediate a protective role, while type II NKT cells suppress antitumor surveillance [124].

There have been conflicting reports regarding the role of NKT cells in asthma. Some studies have implicated NKT cells as being important in the development of allergic AHR [119, 120]. More specifically, type I NKT cells that produce IL-4 and IL-13 were found to be required for the clinical expression of AHR [119], as well as for the maximal production of eosinophilia, Th2 cytokines, and IgE [120]. Jα18$^{-/-}$ and CD1d$^{-/-}$ BALB/c mice were unable to develop AHR, even though they could generate conventional Th2 cells capable of making IL-4 and IL-13 [119]. Moreover, adoptive transfer of type I NKT cells, which produced IL-4 and IL-13, into Jα18$^{-/-}$ mice restored AHR, while administration of IL-13 restored allergic AHR in CD1d$^{-/-}$ mice [119].

By contrast, others have reported that an allergic response can develop in the absence of NKT cells [202, 203]. OVA-sensitized CD1d$^{-/-}$ mice developed eosinophilic-rich inflammation and levels of OVA-specific IgE comparable to WT B6 mice. These findings were supported by Morishima et al. who found that airway eosinophilia, AHR, and OVA-specific IgE levels were no different in Jα18$^{-/-}$ and WT BALB/c mice [204]. Brown et al. assessed the role of NKT cells in allergic AHR using Beta2-microgobulin (β2m) $^{-/-}$ mice, which, due to the disruption of the β2m gene, impairs surface expression of CD1 [202]. They found that β2m$^{-/-}$ mice had no significant difference in lung resistance, airway pressure, IL-4-producing cells, or serum IgE compared to WT mice. A more recent study found that a CD1d-restricted , NK1.1^{+} noninvariant NKT cell was present in β2m$^{-/-}$ mice and was responsible for the development of allergic AHR [205]. Possible explanations for their results include differences in genetic background, experimental protocol for OVA immunization, and the time points used in the evaluations.

Only one other study has examined the role of NKT cells in allergic conjunctivitis. Fukushima et al. found that administration of α-GalCer, which is known to stimulate type I NKT cells, significantly increased Th2 immune responses and infiltration of eosinophils into the conjunctiva when administered at the time of immunization with SRW [192]. However, treatment with α-GalCer just prior to the first challenge resulted in decreased infiltrations of eosinophils into the conjunctiva of SRW-primed mice. This decrease in the late phase reaction of allergic conjunctivitis was associated with an increase in the frequency of $CD4^+CD25^+$ T cells [192].

In order to assess if NKT cells were needed for the full expression of allergic conjunctivitis, we employed two NKT-cell deficient mice: $J\alpha18^{-/-}$ and $CD1d^{-/-}$ mice. $J\alpha18^{-/-}$ BALB/c mice had a reduction in the clinical signs of allergic conjunctivitis as well as in the eosinophils infiltrating into the conjunctiva compared to WT mice. Furthermore, adoptive transfer of NKT cells into $J\alpha18^{-/-}$ B6 mice fully reconstituted the early and late phase of allergic conjunctivitis. These results confirm that NKT cells are needed for the full expression of the early and late phases of allergic conjunctivitis. Since it is not feasible to assess the role of type II NKT cells alone, we used $CD1d^{-/-}$ mice that lack both type I and type II NKT cells. $CD1d^{-/-}$ BALB/c mice also had a decrease in the clinical signs of allergic conjunctivitis and a reduction in the eosinophils infiltrating into the conjunctiva compared to WT mice. It was evident that the clinical severity of allergic conjunctivitis in $J\alpha18^{-/-}$ mice was greater than observed in $CD1d^{-/-}$ mice in both strains, indicating that there might be an additive effect when both type I and type II NKT cells are absent.

The decrease in the early and late phase of allergic conjunctivitis correlated with a decrease in the production of Th2 (IL-4, IL-5, and IL-13) but not Th1 (IFN-γ) cytokines by

SRW-specific Th2 cells. The dampened Th2 response was correlated with high IFN-γ levels that occurred in the NKT-deficient mice. This suggests that additional factors contribute to the decreased Th2 immune responses in these animals. As in allergic AHR, the role of NKT cells in allergic conjunctivitis may be to "license" Th2 cells in the conjunctiva, as adoptively transferred SRW-primed $CD4^+$ T cells were unable to induce allergic conjunctivitis in $J\alpha18^{-/-}$ mice. NKT cells may be contributing to the magnitude of the Th2 immune response or are regulating additional elements in the conjunctivae that are required for the development of allergic conjunctivitis. NKT cells may also contribute to "licensing" by their constitutive production of chemokines or Th2 cytokines that lower the threshold needed for Th2 immune responses to be expressed in situ. AHR, NKT cells are needed at both the afferent and efferent stages of allergic conjunctivitis.

γδ T cells

In allergic asthma, γδ T cells are also necessary for the development of allergic AHR as mice lacking γδ T cells have a decrease in antigen-specific IgE and IgG1, pulmonary IL-5, as well as in eosinophil and T cell infiltration compared to WT mice [137]. All of these responses can be restored by the administration of IL-4 in γδ T cell-deficient mice [137]. It has also been shown that γδ T cells affect the IL-10 levels in the airways, and blocking their function results in an increase in $CD4^+CD25^+$ cells in the lung, suggesting that γδ T cells might inhibit regulatory T cell function [206]. Since the conjunctiva, like the lung, is a mucosal tissue, we hypothesized that γδ T cells play a similar role in allergic conjunctivitis as they do in allergic asthma.

In order to assess if γδ T cells were needed for the full expression of allergic conjunctivitis, γδ T cell-deficient mice were used, as well as mice treated with GL3 antibody, which inhibits γδ T cell function. TCR-$\delta^{-/-}$ mice had decreased clinical signs of allergic

conjunctivitis and reduced granulocytic infiltration into the conjunctiva compared to WT mice. We also found that WT mice treated with GL3 antibody had a reduction in the early and late phase of allergic conjunctivitis. These results confirmed that γδ T cells were needed for the full expression of the early and late phases of allergic conjunctivitis.

Since we show that NKT cells play a role in the development of allergic conjunctivitis [207], the hypothesis that blocking NKT cell activation in TCR-$\delta^{-/-}$ mice would have an additive effect in the suppression of allergic conjunctivitis was tested. These studies demonstrated that the absence of both NKT and γδ innate T cells caused a reduction in the early phase of allergic conjunctivitis, but did not cause further reduction in the late phase allergic conjunctivitis reaction. The additive effect of NKT cells and γδ T cells has been noted in other allergic diseases. Using an adoptive cell transfer approach, Jin et al. found that NKT and γδ T cells were necessary for the acute stages of allergic AHR, but not for the later airway eosinophillic inflammation [208]. Another study on allergic AHR demonstrated that NKT cells secreted IL-4 and IL-13 to produce their effector function, while γδ T cells did not have this effect [209]. These data suggest that collaboration between innate T cells may be important in allergic diseases of mucosal surfaces.

The findings indicate that the decrease in allergic conjunctivitis in mice deficient in innate T cells correlates with a decrease in the production of Th2 (IL-4, IL-5, and IL-13) but not Th1 (IFN-γ) cytokines and suggest that γδ T cells are needed for the optimal generation of Th2 cells. Results from experiments involving the adoptive transfer of SRW-primed $CD4^+$ T cells from TCR-$\delta^{-/-}$ mice into WT mice demonstrated reduced allergic conjunctivitis in WT hosts, providing further support that the optimal development of inflammatory Th2 cells also required an intact γδ T cell repertoire. It was hypothesized that γδ T cells may also be needed for the

expression of Th2-based inflammation at the end stage organ (i.e., the conjunctiva). To address this, we adoptively transferred $CD4^+$ T cells from WT mice into TCR-$\delta^{-/-}$ mice and found that these mice also had a reduction in the expression of allergic conjunctivitis.

How innate T cells aid in the development of allergic conjunctivitis

Innate T cells play a major role in regulating the immune response by bridging the innate and adaptive immune system. Based on our findings, NKT and γδ T cells mediate or amplify the Th2 inflammatory responses in the lymphoid tissue as well as at the ocular surface and are needed for maximal expression of allergic conjunctivitis.

In an allergic response, allergens such as SRW pollen are captured and processed by APC. These APC migrate to the draining LN where they present allergen to T cells in the context of MHC class II molecules. NKT and γδ T cells in the LN may function by providing the initial source of IL-4 needed for Th2 differentiation. These antigen-specific T cells undergo clonal expansion and migrate back to the site of allergen exposure, in this case to the ocular surface. Once at the ocular surface, these $CD4^+$ T cells will produce the Th2 cytokines, IL-4, IL-5, and IL-13, that are needed for an allergic response. Again, NKT and γδ T cells in the eye, and more specifically in the conjunctiva, secrete these Th2 cytokines to generate the milieu needed for the full development of allergic conjunctivitis (Figure 30).

In conclusion, we refined a mouse model of allergic conjunctivitis that mimics what is seen in the clinical setting and determined that innate T cells play an important role in the development and expression of allergic conjunctivitis.

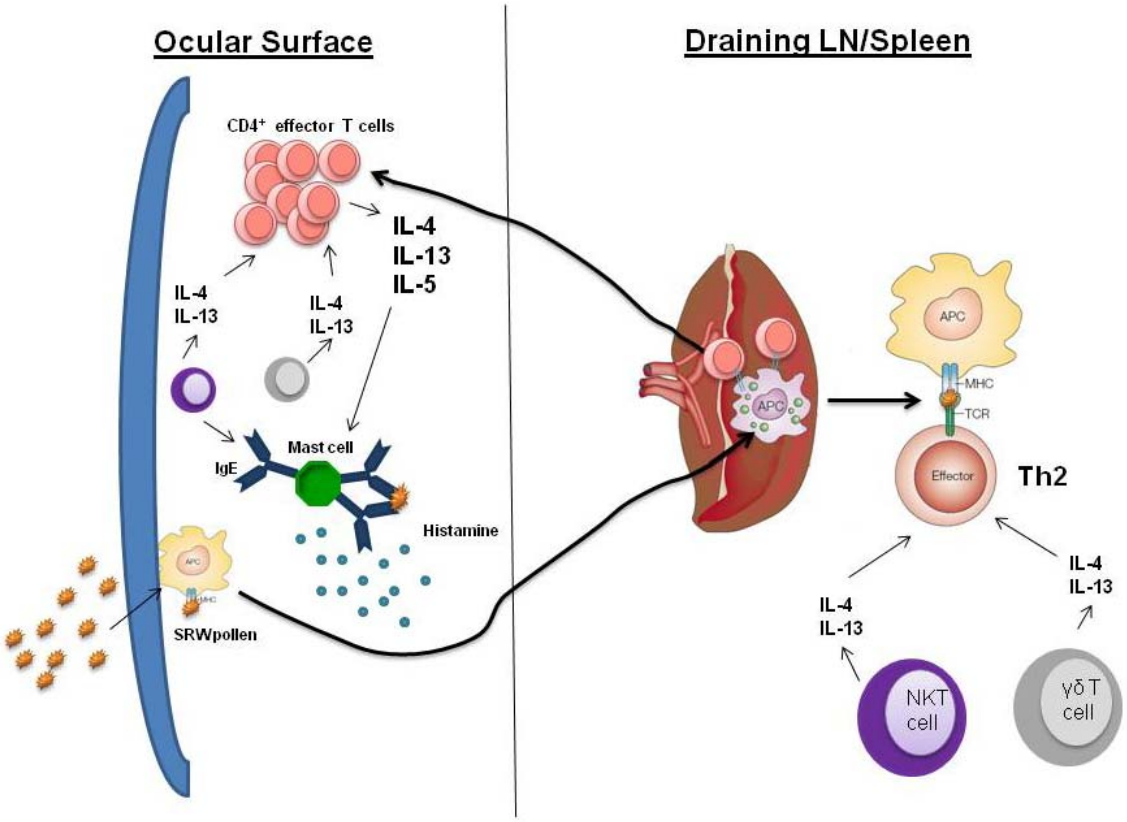


Figure 30. Model: How innate T cells aid in the development of allergic conjunctivitis. NKT and γδ T cells in the LN and/or spleen provide the initial source of IL-4 needed for Th2 differentiation. Additionally, these innate T cells at the ocular surface may secrete IL-4 and IL-13 to promote the Th2 milieu needed for maximal expression of allergic conjunctivitis.

ALLERGIC CONJUNCTIVITIS AND CORNEAL ALLOGRAFT REJECTION

Allograft rejection is a result of type I immune responses and is closely associated with the production of IFN-γ and IL-2. The role of Th2 immune responses in transplantation has been controversial. Th2 cytokines have been associated with graft tolerance in several models including neonatal allograft tolerance. In this model, BALB/c mice primed with splenocytes isolated from the F1 generation of a BALB/c and A/J crossing (CAF1) showed increased tolerance to an A/J skin graft, but not to B6 [210]. Mice with A/J skin grafts had a cytokine profile characterized by high amounts of IL-4 and low amounts of IFN-γ. By contrast, mice with a rejected B6 skin graft had a cytokine profile characterized by high levels of IFN-γ and low levels of IL-4 [210]. In a different model of tolerance, rats that received a renal allograft and were treated with CTLA4-Ig experienced increased graft acceptance. Furthermore, renal allograft rejectors had an immune response characterized by the production of IFN-γ and IL-2; while renal allograft acceptors produced IL-4 and IL-10 [211, 212]. In the setting of heart transplants, C57BL/10 heart grafts transplanted to C3H recipients were rendered tolerant by donor-specific blood transfusions, anti-CD4 antibody pre-treatment, and cyclosporine administration [180]. This tolerance was associated with reduced expression of IFN-γ and IL-2 and an enhanced expression of IL-4 and IL-10. Th2 cytokines have also been associated with Th1-mediated autoimmune diseases. In experimental autoimmune encephalomyelitis (EAE), paralysis was prevented in rats treated with anti-CD4 antibody [213]. In vitro stimulation of $CD4^+$ T cells from these mice in the presence of anti-CD4, led to complete inhibition of IFN-γ. Moreover, after secondary stimulation, synthesis of IL-4 and IL-13 mRNA was enhanced [213]. These data suggests a tolerogenic role for Th2 immune responses in the setting of transplantation and autoimmunity.

Other investigators, however, have found that IL-4 exacerbates graft rejection. One study assessing the effect of Th2 immune response in skin transplants induced Th2 $CD4^+$ and $CD8^+$ T cells *in vitro* by culturing them with IL-4 and anti-IFN-γ [214]. These Th2 cells were adoptively transferred into SCID mice and results showed that $CD4^+$ and $CD8^+$ Th2 cells mediated skin allograft rejection. Furthermore, $CD4^+$, but not $CD8^+$, Th2 cells had distinct eosinophilic infiltration into the skin [214]. In heart transplants, treatment with anti-IL-12 antibody, or with the IL-12 receptor antagonist p40 homodimer, deviated the immune response to a Th2 immune response and resulted in exacerbated graft rejection [215]. Another group studying heart transplants generated Th2 cells *in vitro* by stimulating syngeneic splenocytes with donor alloantigens in the presence of IL-4. Adoptive transfer of these polarized Th2 cells into SCID cardiac allograft recipients resulted in acute rejection within seven to ten days [216]. This was similar to the rejection tempo seen when Th1 cells were adoptively transferred [216].. Interestingly, IL-4 has also been found to have no effect on the outcome of allograft transplants. In one study of heart xenografts, the MST in $STAT4^{-/-}$ and $STAT6^{-/-}$ mice was no different than that of WT BALB/c [217]. Collectively, these studies suggest that Th2 immune responses have a deleterious effect in the setting of allograft transplantation.

In corneal transplantation, patients with acvascular corneas have a success rate as high as 90% [177]. Vascularization of the cornea, re-grafts, bilateral grafts, and increase in the presence of LCs are all factors that decrease the rate of success of a corneal transplant [218]. As previously mentioned, rejection of a corneal transplant has been established as being a Th1-mediated immune response. Th1 immune responses are characterized by the production of IFN-γ and IL-2 as well as the development of DTH responses directed towards donor antigens leading to corneal allograft rejection [219, 220].

In the classical immunology paradigm IFN-γ cross-regulates IL-4 [219]. Initial studies in which the immune response was deviated to a Th2 response prior to corneal transplantation by the induction of allergic conjunctivitis exacerbated, rather than diminished, graft rejection [80, 98, 99]. Interestingly, this rejection was not simply due to local inflammation caused by the allergen since rejection was increased in SRW pollen-induced allergic conjunctivitis even when the graft was placed on the unchallenged eye [80]. More importantly, mice with allergic AHR that received a corneal allograft also experienced an increase in graft rejection [98]. These studies establish that allergic diseases cause a systemic disturbance in the alloimmune response that is responsible for the exacerbation in graft rejection. Other investigators have looked at the effect of IL-4 on corneal transplantation using a sheep model. This group found that gene transfer of cDNA encoding IL-4 on ovine corneal endothelium induced graft rejection that was characterized by eosinophilia and inflammation [181]. This was in sharp contrast to the prolonged corneal graft survival seen when cDNA encoding IL-12-p40 was induced [181]. In humans, one study assessed if there was any evidence for Th2-mediated rejection in patients with keratoconus who received a corneal transplant. This study found that atopic keratoconus patients who underwent graft rejection had significantly greater density of eosinophils in their corneal tissue compared to patients that did not have pre-existing Th2 bias [96]. This suggests that the rejection seen in atopic patients may be similar to the rejection seen in the murine model. More evidence that Th2 immune responses exacerbate graft rejection came from Cunnusamy et al. who found that mice treated with both anti-IFN-γ and anti-IL-17 had a preponderance of Th2 responses, which in turn, exacerbated graft rejection [169].). Furthermore, adoptive transfer of these Th2 $CD4^+$ T cells into T cell-deficient mice led to 100% corneal allograft rejection [160]. These results were in accordance with earlier findings in IFN-$\gamma^{-/-}$ BALB/c who experienced

increased corneal allograft rejection that was characterized by eosiniphillic infiltrate in the corneal bed, increased production of IL-4, IL-5, and IL-10 in the draining LN, and positive DTH to donor antigen [221]. Additionally, STAT6$^{-/-}$ mice, which cannot generate Th2 cytokines, that were depleted of IL-17 did not experience exacerbated graft rejection [160]. Based on these observations, this study set out to examine why allergic diseases, and more importantly why allergic conjunctivitis, exacerbate corneal allograft rejection.

Allergic conjunctivitis does not affect the generation of ACAID

The first aim this study addressed was if allergic conjunctivitis affected the generation and expression of ACAID. Antigens introduced into the AC chamber of the eye elicit a form of immune tolerance known as ACAID, which leads to the suppression of DTH responses [68]. Abolishing ACAID by splenectomy dramatically increases the incidence of corneal allograft rejection [59]. Conversely, introducing alloantigens into the AC to induce ACAID prior to corneal transplantation enhances graft survival [70]. Initially, we hypothesized that the increased incidence of corneal allograft rejection in mice with allergic conjunctivitis was due to the inability of these recipient mice to induce ACAID. Therefore, the ability of mice with allergic conjunctivitis to induce ACAID was examined. To address the argument that allergic conjunctivitis abolishes ACAID because of local disturbances caused by the SRW pollen, allergic conjunctivitis was induced in one eye while alloantigens were injected into the other. Results indicate that in mice, allergic conjunctivitis does not affect the development of ACAID and these mice are able to suppress DTH responses.

Allergic Conjunctivitis does not cause a qualitative or quantitative difference in the alloimmune response

Like mice with allergic diseases, human hosts with vascularized corneal graft beds experience an increased incidence of corneal allograft rejection [80, 222, 223]. Vascularization of the corneal graft bed promotes vascular as well as lymphatic formation that is believed to increase migration of APC from the graft bed to the regional lymphoid tissue [85]. It is also thought to facilitate the migration of effector T cells back to the graft site. Animal studies have found that unlike corneal allografts transplanted onto normal, avascular graft beds, mice with vascularized graft beds generate donor-specific CTL [222]. We tested the hypothesis that like these high-risk hosts, mice with allergic conjunctivitis would generate CTL; however, results showed that no CTL are generated in allergic rejectors. Unlike the previous reports, we were unable to show CTL activity even in graft rejectors who had pre-vascularized graft beds.

While our findings show that there was no qualitative difference in the alloimmune response of allergic rejectors, there was the possibility that there was a quantitative difference. It is known that corneal allograft rejection is dependent upon $CD4^+$ T cells [147], therefore experiments were performed to determine if the increased rejection in mice with allergic conjunctivitis was due to an enhanced response by their $CD4^+$ T cells. Our results show that the sensitization protocol used to induce allergic conjunctivitis did not affect the $CD4^+$ T cell alloimmune response.

Allergic conjunctivitis does not affect Treg development

Our group and several others have shown the importance of $CD4^+CD25^+$ Tregs in corneal allograft survival [171, 172]. Corneal allograft recipients treated with anti-CD25 antibody

experienced and exacerbation of corneal allograft rejection [171]. Moreover, adoptive transfer of Tregs into graft recipients increases corneal allograft survival [172]. Further dissection of the Treg population revealed that there were no differences in the frequency of Tregs between graft acceptors versus graft rejectors. Tregs from graft acceptors have increased mean fluorescence intensity (MFI) of Foxp3 compared to graft rejectors [172]. The question remains as to why the suppressive function of Tregs is overridden when mice are subjected to allergic inflammation.

In our investigation, the *in vitro* suppression assays confirm results found by other groups showing that $CD4^+CD25^+$ Tregs isolated 21 days post-transplantation from graft acceptors can suppress the proliferation of effector T cells [169, 172]. Interestingly, the addition of IL-4 to the culture blocked suppression by Tregs. Adding IL-5 or IL-13 did not affect the suppression asserted by Tregs. In the suppression assay we utilized, the cell proliferation detected can potentially come from multiple sources. There was the possibility that IL-4 could be causing Tregs to proliferate *in vitro* as has been reported by other groups [224]. Accordingly, we prevented any possible proliferation caused by the Treg population by treating the Treg suspension with mitomycin-C. These *in vitro* suppression assays confirmed that the proliferation detected originally came from the $CD4^+$ effector T cell population and not from the Tregs. Our group has shown that Tregs from corneal allograft acceptors exert their suppression through a contact-dependent manner [169]. Furthermore, simultaneous blockade of GITR, CTLA-4, and membrane bound TGF-β *in vitro* led to a significant reduction in the suppression of $CD4^+$ T cell activation caused by Tregs [169], suggesting that proliferation of Tregs is not necessary for them to enforce their suppressive function.

The results from the *in vitro* suppression assays were confirmed using a LAT assay, which measures suppression *in vivo*. As has been previously shown, Tregs from graft acceptors

suppressed DTH responses to donor alloantigens [169]. Our study is the first to show that IL-4 inhibits the suppression produced by Tregs. Similar to our results in the *in vitro* suppression assays, IL-5 and IL-13 did not affect suppression of DTH by Tregs. Taken together, our data show for the first time how a Th2 cytokine, IL-4, can block suppression produced by Tregs in a corneal transplantation setting.

Next, our investigation tested our original hypothesis that IL-4 could be affecting the Treg population by inhibiting their development or by rendering them unable to suppress effector T cells (Figure 31). Studies assessing the effect of IL-4 on $CD4^+CD25^+$ Treg function have been extremely variable. IL-4 has been shown to prevent spontaneous apoptosis of Tregs and the decline of Foxp3 mRNA *in vitro*, as well as enhancing CD25 expression on Tregs [225]. IL-4 and IL-13 have also been shown to induce the generation of $Foxp3^+CD25^+$ Tregs from $CD25^-$ precursor T cells [226]. Both studies show that IL-4 enhances the ability of Tregs to inhibit the proliferation of effector T cells as well as the production of IFN-γ [225, 226].

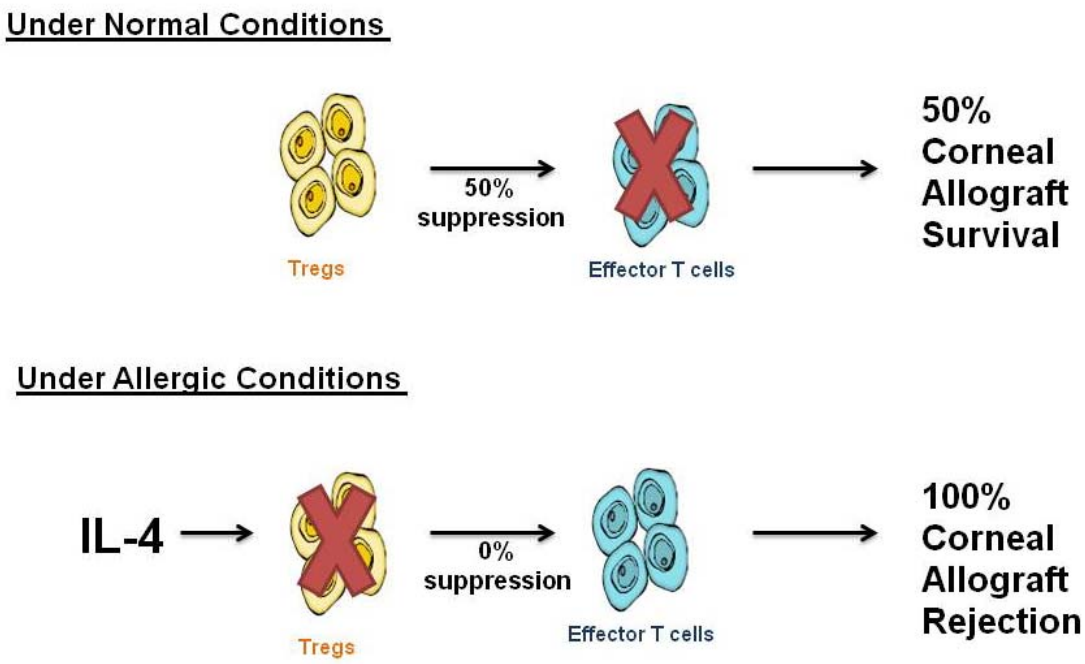


Figure 31. IL-4 affects Treg development. Our original hypothesis proposed that the IL-4 produced during allergic diseases affected the development and function of Tregs, allowing for effector T cells to mediate rejection.

By contrast, one study found that TGF-β-induced Foxp3 levels were maintained by blocking IL-4 or by STAT6 gene deletion [227]. Furthermore, having IL-4 present at the time of T cell priming was found to inhibit Foxp3 expression, and blocking IL-4 promoted $Foxp3^+$ Treg differentiation [228, 229]. Further dissection revealed that this inhibition was mediated by binding of GATA3 to the Foxp3 promoter [229]. Inhibition of $Foxp3^+$ Treg induction required full activation of the central signaling pathways for Th1/Th2 lineage differentiation (T-bet, STAT1, and STAT6) [228]. One study found that while IL-4 was required for maintenance of Foxp3 expression in Tregs and to promote their proliferation, it compromised Treg-mediated suppression [224].

While $CD4^+CD25^+$ Tregs play an important role in corneal allograft survival, there is also evidence that $CD8^+$ Tregs play a role. For one, injection of donor alloantigenic cells into the AC before corneal transplantation induces ACAID and results in a significant enhancement of corneal allograft survival in both the rat and mouse models of penetrating keratoplasty [69, 70]. In addition, hosts that have received AC injections to induce ACAID and mice with long-term surviving corneal allografts have suppressed DTH responses to donor alloantigens. Furthermore, abrogating the induction of ACAID by splenectomy or deletion of NKT cells or γδ T cells greatly increases corneal allograft rejection [59, 71, 72]. Th2 cytokines have also been shown to affect the function of $CD8^+$ Tregs. $CD8^+CD25^+$ Treg clones were generated from human thymocytes and their ability to suppress both Th1 and Th2 clones was investigated. $CD8^+CD25^+$ Tregs suppressed the proliferation of Th1 clones, but had lower suppressive activity on the proliferation of Th2 clones [230]. The partial suppression on Th2 cell proliferation was further reduced with the addition of IL-4, while the addition of anit-IL-4 increased the suppression [230]. In a model of oral tolerance, $CD8^+$ T cells were shown to be responsible for mediating

suppression of DTH responses, as well as Th1 and Th17 cytokine production [231]. Inducing oral tolerance in mice prior to inducing allergic lung inflammation did not result in suppression of this Th2-mediated inflammation. Antigen-fed mice had a robust inflammatory response, similar to PBS-fed mice, that consisted of eosinophil-rich infiltrates in the peri-bronchiolar and peri-vascular regions of the lung, and hypertrophy of the bronchiolar epithelium [231]. The study presented here does not address if Th2 cytokines affect $CD8^+$ Tregs and their support in corneal allograft survival; however, it is necessary to assess the effect of Th2-mediated inflammatory responses on this Treg population.

We first determined if allergic conjunctivitis affected the generation of Tregs. Tregs were isolated from individual allergic rejectors and their suppressive function was assessed. The suppressive profile of Tregs from mice with and without allergic conjunctivitis was not statistically different. This indicated that in corneal transplantation, allergic conjunctivitis, and more specifically IL-4, does not affect Treg development. While allergic conjunctivitis, namely IL-4, does not affect the global Treg population, it could be affecting antigen-specific, induced Tregs. Studies in which the stimulation in the suppression assays is antigen-specific need to be performed to address this possibility.

IL-4 renders effector T cells resistant to Treg suppression

Our study investigated the hypothesis that IL-4 rendered effector T cells resistant to Treg suppression (Figure 32). Effector T cells, unable to respond to IL-4 (IL-4R$\alpha^{-/-}$), were utilized in a suppression assay and were found to be amenable to Treg suppression even in the presence of IL-4. This provides evidence that IL-4 does not directly affect Tregs since IL-4R$\alpha^{-/-}$ effector T cell proliferation is suppressed by $CD4^+CD25^+$ Tregs, even in the presence of IL-4. Pillemer et

al. found that in mice with allergic AHR, IL-4 rendered effector T cells resistant to suppression by natural Tregs [224]. The suppression was restored even in the presence of IL-4 when STAT6$^{-/-}$ T cells were utilized. Furthermore, when STAT6 was introduced back into these cells, resistance to Treg suppression was restored. In the setting of corneal transplantation, our results are the first showing that IL-4 renders effector T cells resistant to Treg suppression, and as a result, mice with allergic conjunctivitis experience an increased incidence of graft rejection.

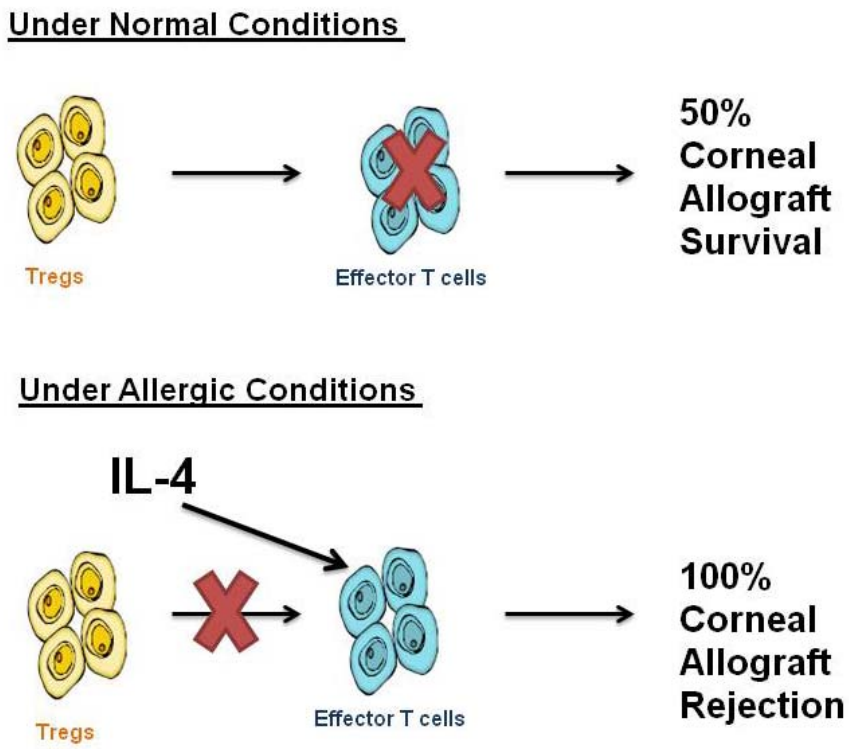


Figure 32. IL-4 renders effector T cells resistant to Treg suppression. Our second hypothesis proposed that under normal, non-allergic conditions, Tregs suppress effector T cell proliferation and prevent graft rejection. However, during active allergic diseases, IL-4 renders effector T cells resistant to suppression by Tregs, allowing them to mediate graft rejection.

Our results showing that IL-4 renders effector T cells resistant to Treg suppression led us to hypothesize that IL-4Rα$^{-/-}$ mice with allergic conjunctivitis would have increased corneal allograft survival compared to WT mice with allergic conjunctivitis. IL-4R signaling is critical for the development and maintenance of Th2 cells [232-234]. The receptors for IL-4 and IL-13 share a common IL-4Rα chain responsible for the signal transductions critical for this phenotype [235]. Therefore, we first had to establish that IL-4Rα$^{-/-}$ mice developed allergic conjunctivitis. Surprisingly, both the early and late phase of allergic conjunctivitis in IL-4Rα$^{-/-}$ mice developed the same magnitude as WT mice. Several groups have hinted that signaling through the IL-4Rα chain is not essential for development of a Th2 phenotype. Adoptive transfer of IL-13-producing CD4^{+} T cells into IL-4Rα$^{-/-}$ mice were able to induce allergic AHR, mucus hypersecretion, eotaxin production, and eosinophilia in the lungs [236]. This development did not occur if the CD4^{+} T cells adoptively transferred were incapable of producing IL-13. Studies of *Nippostrongylus brasiliensis*, a gastrointestinal nematode parasite of rats, have reported that CD4^{+} T cells from IL-4Rα$^{-/-}$ animals still produce IL-4 during infection [237]. Further analysis of these CD4^{+} T cells strongly suggests that the IL-4-producing cells are NKT cells. This coincides with our findings in allergic conjunctivitis suggesting that an innate T cell population such as NKT cells provides a source of IL-4 needed for maximal expression of allergic disease [189, 191]. Furthermore, stimulation of CD4^{+} T cells from IL-4Rα$^{-/-}$ mice through their TCR stimulates the release of IL-4 [238]. In turn, this small amount of IL-4 is sufficient to cause differentiation of CD4^{+} T cells into Th2 cells. Thus, production of IL-4 can occur in the absence of IL-4R signaling.

Establishing that IL-4Rα$^{-/-}$ mice developed allergic conjunctivitis comparable to WT mice was critical for assessing our hypothesis. Accordingly, allergic conjunctivitis was induced

in IL-4Rα$^{-/-}$ mice prior to receiving corneal allografts. Corneal allograft survival in the allergic IL-4Rα$^{-/-}$ mice was the same 50% as is routinely observed in non-allergic WT mice. Our study also found that treating WT mice with anti-IL-4 antibody the day they received a corneal transplant also restored survival to the normal 50% level, even though the mice received continuous exposure to SRW pollen. These findings confirm that allergic conjunctivitis does not exacerbate corneal allograft rejection unless mice are able to respond to IL-4. The possible therapeutic effects of these findings will be discussed later.

It is important to explore why IL-4 may cause effector T cells to become resistant to Treg suppression. In allergic asthma patients, effector T cells were found to have a higher proliferative response when stimulated through their TCR compared to healthy controls [196]. Furthermore, these effector T cells were resistant to suppression by TGF-β and IL-10. Mitogen-activated protein kinase (MAPK) family members play an important role in T cell proliferation; therefore, it was possible that this pathway was being affected [239]. The resistance to suppression produced by TGF-β and IL-10 correlated with increased expression of MEK1, the upstream activator of ERK1/2. Furthermore, inhibiting MEK1 resulted in TGF-β being more effective at suppressing T cell proliferation in asthmatic patients at a level that was comparable to that observed in non-allergic subjects [196]. There is the possibility that the up-regulation of MEK1 renders effector T cells resistant by affecting the surface expression of several molecules needed for Tregs to mediate their suppressive function. Tregs constitutively express Foxp3 and GITR mRNA, as well as CTLA-4 [240, 241]. As previously mentioned, in corneal transplantation Treg suppression of effector T cells is dependent on CTLA-4, GITR, and membrane-bound TGF-β [169]. Other groups have confirmed that Treg suppressive function is related to the expression of both CTLA-4 and TGF-β that inhibits the expression of IL-2 receptor on effector T cells [230,

241]. If MEK1 is up-regulated in effector T cells in hosts with allergic conjunctivitis, this could be enhancing the expression of IL-2 receptor. This over-expression of IL-2 receptor may counter-act the suppression mediated by Tregs. Treg inhibition of $CD4^{+}CD25^{-}$ T cell proliferation has also been shown to be abrogated by anti-CTLA-4 mAb or by its Fab fragment [242]. CTLA-4 functions by interacting with CD28 on an effector T cell resulting in inhibition of proliferation. The resistance of effector T cells under allergic conditions could be due to an altered expression in CD28 compared to the expression seen on effector T cells under non-allergic conditions. Interestingly, CTLA-4-deficient Tregs could still suppress through TGF-β and IL-10 *in vitro* and *in vivo* [243, 244]. It is possible the TGF- β receptor or IL-10 receptor expression on an effector T cell is down-regulated under allergic conditions. This down-regulation could affect their ability to be suppressed. Further studies need to be conducted to determine the expression of these molecules on effector T cells under allergic and non-allergic conditions.

It is interesting that although MEK1 is up-regulated in asthmatic patients, this up-regulation is not seen in patients with allergic rhinitis, suggesting that a Th2 phenotype alone does not lead to increased MEK1 expression [196]. Similarly, mice with subcutaneous immediate hypersensitivity reactions who receive a corneal allograft do not experience increased rejection [245]. Further dissection of their MEK1 expression needs to be conducted in our model of corneal transplantation.

Another possible mechanism as to how allergic diseases render effector T cells resistant to Treg suppression may involve transcription factors that are important in Th2 differentiation: STAT6 and GATA-3. One report has shown that Tregs expressing membrane bound TGF-β induced Foxp3 expression in naïve effector T cells, allowing these T cells to have a suppressive

phenotype [246]. Interestingly, both STAT6 and GATA-3 have been shown to directly bind to the Foxp3 promoter, thus blocking TGF-β1-induced Foxp3 expression [227, 229]. The induction of Foxp3 could compete with the opposing effects of STAT6 and GATA-3.

This study also found that resistance of effector T cells to Treg suppression is transient since isolating mice with allergic conjunctivitis from SRW pollen exposure restores the incidence of corneal allograft survival to normal levels. This observation hints as to the kinds of therapies needed for allergic patients undergoing a corneal transplant. To summarize, this study establishes that allergic diseases, like many other factors, abolish immune privilege (Table 4). It also provides evidence that the mechanism by which allergic diseases increase corneal allograft rejection is due to an IL-4-induced resistance of effector T cells to Treg suppression.

Factors	Possible Mechanisms	References
Vascularization	Generation of donor-specific CTL; Coincides with lymph vessel formation believed to increase APC and effector T cell trafficking	Ksander et al. 1996 Dietrich et al. 2010 Amescua et al. 2008
Langerhans Cells	Increases immunogenicity of allograft	Steinman et al.1991 Callanan et al. 1988 He et al. 1996
Allergic Diseases	Promotes the secretion of IL-4, IL-5, and IL-13; IL-4 renders effector T cells resistant to Treg suppression	Present study

Table 4. Factors that affect immune privilege. The eye enjoys an immune privilege not seen in other forms of tissue transplantations. However, listed above are some conditions that cause the eye to lose its immune privilege and exacerbate corneal transplant rejection.

THERAPEUTIC IMPLICATIONS

The current therapy for keratoplasty relies on topical application of corticosteroids [141]. Long-term use of this carries the risk for development of glaucoma and cataracts. With the negative side-effects of current therapies, developing treatments that enhance tolerance is necessary.

Our study provided insight into potential treatments and therapies for allergic patients that are undergoing a corneal transplantation. First, this study found that IL-4 inhibited Treg suppressive function that is necessary for corneal allograft survival. This finding is similar to results found by other investigators who have examined Tregs in a Th2 setting [224, 227-229]. Further dissection revealed that IL-4 rendered effector T cells resistant to Treg suppression. Treating allergic grafted mice with anti-IL-4 restored graft survival to the normal 50% level of acceptance. Based on these observations, it would be interesting to see if topical or systemic treatment with anti-IL-4 could increase graft survival in allergic hosts.

In allergic asthma patients, resistance of effector T cells to Treg suppression was associated with MEK1 upregulation. The MEK1 inhibitor U0126 has been shown to reduce airway inflammatory cell infiltration, Th2 cytokine levels, AHR, mucus production, and IgE levels [247]. Therefore, using this MEK1 inhibitor (preferably topically) may also decrease the incidence of rejection in allergic hosts.

This study also found that isolating allergic hosts from their allergens for a period as short as two weeks, can also increase graft survival. These findings are very promising as a potential therapy that does not involve immunosuppressants. It will be important to perform similar studies to better define the kinetics in humans. While this is a promising avenue, this kind of isolation is only feasible for patients who suffer from seasonal outdoor allergies. Seasonal

allergies are most frequently caused by grass, tree, and weed pollens and outdoor molds which peak at different times of the year [110, 248]. Other forms of allergies occur year round and are caused by house dust mites, animal dander, insects and indoor moulds [248]. In these patients, isolation from their allergens is not a promising avenue.

Future Studies

The work presented in this study has provided insight into how allergic diseases exacerbate corneal allograft rejection. Under allergic inflammation, the IL-4 being produced renders effector T cells resistant to Treg suppression. Further experiments need to be conducted to determine what factors are rendering effector T cells resistant to Treg suppression.

The study by Liang and colleagues provides insights into the mechanism [196]. Conditions associated with allergic conjunctivitis, namely IL-4, may cause an upregulation of MEK1 in effector T cells. MAPK enhances the transcriptional activation of protein-1, ATF2, and c-Jun which leads to gene expression, cytokine production, differentiation, and proliferation of inflammatory cells [249, 250]. This tyrosine signaling cascade may be upregulated in allergic conjunctivitis. Therefore, MEK1 expression needs to be assessed in naïve $CD4^+$ T cells cultured with IL-4 and compared to $CD4^+$ T cells that have not been exposed to IL-4. In addition, MEK1 expression in $CD4^+$ T cells from mice with allergic conjunctivitis that have a corneal allograft will need to be assessed and compared to non-allergic controls (both graft acceptors and graft rejectors). If MEK1 expression is upregulated in an IL-4-rich environment, then studies can be done to determine if the effect of IL-4 can be reversed. For example, an *in vitro* suppression assay can be performed in which Tregs from graft acceptors are co-cultured with effector T cells with IL-4 and the MEK1 inhibitor, U0126 [247]. If the resistance to suppression by the effector T cells involves this tyrosine signaling cascade, then this inhibitor should reverse that phenotype. Furthermore, our study found that eliminating IL-4 either by gene deletion of the IL-4 receptor or by treatment with anti-IL-4 antibody would restore the survival rate to the normal 50%. MEK1 inhibitor U0126 can also be used *in vivo* [247]. Therefore, mice with allergic conjunctivitis can be treated with this inhibitor at the time that they receive a corneal transplant to determine if

graft survival can be restored to normal. If these results indicate that corneal transplantation is restored to normal levels, using a MEK1 inhibitor or anti-IL-4 antibody could be a promising therapy for patients.

As noted earlier, there are two phases in the development of allergic diseases: an early phase and a late phase reaction. The early phase reaction occurs within minutes of allergen exposure and is initiated when the allergen crosslinks specific IgE antibodies bound via FcεRI receptors on the surface of ocular mast cells resulting in the release of histamines, leukotrienes, proteases, prostaglandins, and cytokines [100, 101]. One study found that histamine causes a decrease in CD25 and Foxp3 expression on Tregs [251]. In addition to this down-regulation, co-culturing Tregs with activated mast cells inhibited the suppression of effector T cell proliferation mediated by Tregs. Further analysis found that histamine directly inhibited Treg suppressor function as opposed to rendering effector T cells resistant to Treg-mediated suppression. This inhibitory effect of histamine on Treg function was mediated exclusively through H1 receptors. Future studies can examine if Tregs induced by corneal allografts are affected by histamine by performing i*n vitro* suppression assays in which Tregs are co-cultured with effector T cells in the presence or absence of histamine.

While trying to optimize the allergic conjunctivitis protocol, we found that NKT cells and γδ T cells are necessary for maximal expression. Recently, there was an interesting observation made in allergic asthma patients by Nguyen and colleagues. $CD4^+$ iNKT cells from allergic asthma patients had increased cytotoxicity against Tregs [252]. Cytotoxicity of these iNKT cells in severe allergic asthma subjects was higher than in mild-to-moderate allergic asthma subjects. Furthermore, oral corticosteroid treatment abrogated increased cytotoxicity of iNKT cells against Tregs. Future studies can look at this population in allergic rejectors to assess if their NKT cells

have increased cytotoxicity against Tregs. While preliminary data in our lab reveals there are no differences in total Treg numbers between non-allergic rejectors, allergic rejectors, and graft acceptors, this potential killing phenomenon of NKT cells against Tregs can be occurring at the graft site or to a specific sub-population of Tregs that are induced and antigen-specific. If these NKT cells have increased cytotoxity to Tregs, *in vivo* studies can be performed to determine if topical treatment with anti-CD1d antibody can counteract this effect and restore corneal allograft survival to normal levels. This again may be a potential therapy for allergic patients that will receive a corneal transplant.

Studies presented here did not dissect any potential effects IL-4 may be having on Tregs even though the suppressive profile of Tregs from both allergic and non-allergic grafted mice was comparable. Allergic inflammation may still be having an effect on Tregs. Several groups have shown that IL-4 does in fact have a deleterious effect on Tregs [225, 226]. Tregs induced during corneal transplantation mediate their suppression through the cell-membrane molecules CTLA-4, GITR, and mTGF-β [169]. It is possible that these molecules may be down-regulated in mice with allergic conjunctivitis. Experiments need to be performed to examine the expression of these molecules on Tregs in mice with allergic conjunctivitis. Even though I found no differences in the suppressive function of total Tregs in mice with and without allergic conjunctivitis, I did not specifically look at induced, antigen-specific Tregs. The function and expression of the suppressive molecules on this Treg population may still be affected by allergic conjunctivitis. Conversely, the receptor for these suppressive molecules could be affected on an effector T cell. As mentioned earlier, the surface expression of CD28, IL-2 receptor, TGF-β receptor, and IL-10 receptor need to be assessed on an effector T cell under allergic and non-allergic conditions.

In conclusion, this study described investigated the role of allergic conjunctivitis in corneal allograft rejection. Treg development does not seem to be affected by allergic conjunctivitis. This study has provided evidence that Th2 cytokines, namely IL-4, generated during allergic conjunctivitis render effector cells resistant to the actions of Tregs. Furthermore, allergic conjunctivitis does not exacerbate corneal allograft rejection unless host effector T cells are able to respond to IL-4. These findings will pave the way for developing therapies aimed at improving the survival of corneal transplants in patients suffering from allergic diseases.

REFERENCES

1. DelMonte, D.W. and T. Kim, *Anatomy and physiology of the cornea.* Journal of Cataract and Refractive Surgery, 2011. **37**(3): p. 588-98.
2. Hanna, C., D.S. Bicknell, and J.E. O'Brien, *Cell turnover in the adult human eye.* Archives of Ophthalmology, 1961. **65**: p. 695-8.
3. Baddon, A., et al., *A review of allograft ophthalmic tissue in eye surgery.* Cell Tissue Bank, 2010. **11**(1): p. 29-38.
4. Srinivas, S.P., *Cell signaling in regulation of the barrier integrity of the corneal endothelium.* Experimental Eye Research, 2012. **95**(1): p. 8-15.
5. Holland, J.K.M.M.E., *Basic science and pathophysiologic responses.* Cornea: Fundamentals of Cornea and External Disease, 1997. **1**: p. 1-551.
6. Fu, H., D.F. Larkin, and A.J. George, *Immune modulation in corneal transplantation.* Transplant Rev (Orlando), 2008. **22**(2): p. 105-15.
7. Moffatt, S.L., V.A. Cartwright, and T.H. Stumpf, *Centennial review of corneal transplantation.* Clin Experiment Ophthalmol, 2005. **33**(6): p. 642-57.
8. Kissam, R., *Ceratoplastic in man.* New York Journal of Medicine, 1844. **2**: p. 281-289.
9. America, E.B.A.o., *2009 Eye Banking Statistical Report.* EBAA, 2009: p. 1-17.
10. Niederkorn, J.Y., *The immune privilege of corneal allografts.* Transplantation, 1999. **67**(12): p. 1503-8.
11. Price, F.W., Jr., W.E. Whitson, and R.G. Marks, *Graft survival in four common groups of patients undergoing penetrating keratoplasty.* Ophthalmology, 1991. **98**(3): p. 322-8.
12. Dandona, L., et al., *Survival analysis and visual outcome in a large series of corneal transplants in India.* British Journal of Ophthalmology, 1997. **81**(9): p. 726-31.
13. Price, F.W., Jr., et al., *Five-year corneal graft survival. A large, single-center patient cohort.* Archives of Ophthalmology, 1993. **111**(6): p. 799-805.
14. Williams, K.A., et al., *Factors predictive of corneal graft survival. Report from the Australian Corneal Graft Registry.* Ophthalmology, 1992. **99**(3): p. 403-14.
15. Administration, H.R.a.S., *OPTN/SRTR Annual Report.* 2008: p. 1-4.
16. CCTSR, *The collaborative corneal transplantation studies (CCTS). Effectiveness of histocompatibility matching in high-risk corneal transplantation. The Collaborative Corneal Transplantation Studies Research Group.* Arch Ophthalmol, 1992. **110**(10): p. 1392-403.
17. *The collaborative corneal transplantation studies (CCTS). Effectiveness of histocompatibility matching in high-risk corneal transplantation. The Collaborative Corneal Transplantation Studies Research Group.* Arch Ophthalmol, 1992. **110**(10): p. 1392-403.
18. Sonoda, Y. and J.W. Streilein, *Orthotopic corneal transplantation in mice--evidence that the immunogenetic rules of rejection do not apply.* Transplantation, 1992. **54**(4): p. 694-704.
19. Callanan, D., J. Peeler, and J.Y. Niederkorn, *Characteristics of rejection of orthotopic corneal allografts in the rat.* Transplantation, 1988. **45**(2): p. 437-43.
20. Auchincloss, H., Jr., et al., *T-cell subsets, bm mutants, and the mechanisms of allogeneic skin graft rejection.* Immunologic Research, 1989. **8**(2): p. 149-64.
21. Niederkorn, J.Y., *Ocular Immune Privilege: Nature's Strategy for Preserving Vision.* Science and Medicine, 2003. **9**(6): p. 320-331.

22. Medawar, P.B., *Immunity to homologous grafted skin; the fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye.* Br J Exp Pathol, 1948. **29**(1): p. 58-69.
23. Maumenee, A.E., *The influence of donor-recipient sensitization on corneal grafts.* American Journal of Ophthalmology, 1951. **34**(5:2): p. 142-52.
24. Ksander, B.R., Y. Sano, and J.W. Streilein, *Role of donor-specific cytotoxic T cells in rejection of corneal allografts in normal and high-risk eyes.* Transplant Immunology, 1996. **4**(1): p. 49-52.
25. Niederkorn, J.Y., *High-risk corneal allografts and why they lose their immune privilege.* Curr Opin Allergy Clin Immunol, 2010. **10**(5): p. 493-7.
26. Raju, S. and J.B. Grogan, *Allograft implants in the anterior chamber of the eye of the rabbit. Early vascularization and sensitization of the host.* Transplantation, 1969. **7**(6): p. 475-83.
27. Franklin, R.M. and R.A. Prendergast, *Primary rejection of skin allografts in the anterior chamber of the rabbit eye.* Journal of Immunology, 1970. **104**(2): p. 463-9.
28. Kaplan, H.J. and J.W. Streilein, *Immune response to immunization via the anterior chamber of the eye. II. An analysis of F1 lymphocyte-induced immune deviation.* Journal of Immunology, 1978. **120**(3): p. 689-93.
29. Kaplan, H.J. and J.W. Streilein, *Immune response to immunization via the anterior chamber of the eye. I. F. lymphocyte-induced immune deviation.* Journal of Immunology, 1977. **118**(3): p. 809-14.
30. Kaplan, H.J., J.W. Streilein, and T.R. Stevens, *Transplantation immunology of the anterior chamber of the eye. II. Immune response to allogeneic cells.* Journal of Immunology, 1975. **115**(3): p. 805-10.
31. Streilein, J.W., *Ocular immune privilege: therapeutic opportunities from an experiment of nature.* Nat Rev Immunol, 2003. **3**(11): p. 879-89.
32. Jager, M.J., *Corneal Langerhans cells and ocular immunology.* Regional Immunology, 1992. **4**(3): p. 186-95.
33. Dana, M.R., J. Yamada, and J.W. Streilein, *Topical interleukin 1 receptor antagonist promotes corneal transplant survival.* Transplantation, 1997. **63**(10): p. 1501-7.
34. Abi-Hanna, D., D. Wakefield, and S. Watkins, *HLA antigens in ocular tissues. I. In vivo expression in human eyes.* Transplantation, 1988. **45**(3): p. 610-3.
35. Lampson, L.A. and C.A. Fisher, *Weak HLA and beta 2-microglobulin expression of neuronal cell lines can be modulated by interferon.* Proceedings of the National Academy of Sciences of the United States of America, 1984. **81**(20): p. 6476-80.
36. Le Bouteiller, P., *HLA class I chromosomal region, genes, and products: facts and questions.* Critical Reviews in Immunology, 1994. **14**(2): p. 89-129.
37. Ljunggren, H.G., et al., *The RMA-S lymphoma mutant; consequences of a peptide loading defect on immunological recognition and graft rejection.* International Journal of Cancer. Supplement, 1991. **6**: p. 38-44.
38. Niederkorn, J.Y., et al., *Expression of a nonclassical MHC class Ib molecule in the eye.* Transplantation, 1999. **68**(11): p. 1790-9.
39. Rouas-Freiss, N., et al., *Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytolysis.* Proceedings of the National Academy of Sciences of the United States of America, 1997. **94**(21): p. 11520-5.

40. Niederkorn, J.Y., *See no evil, hear no evil, do no evil: the lessons of immune privilege.* Nature Immunology, 2006. **7**(4): p. 354-359.
41. Taylor, A.W., *Ocular immunosuppressive microenvironment.* Chemical Immunology, 1999. **73**: p. 72-89.
42. Taylor, A.W., J.W. Streilein, and S.W. Cousins, *Alpha-melanocyte-stimulating hormone suppresses antigen-stimulated T cell production of gamma-interferon.* Neuroimmunomodulation, 1994. **1**(3): p. 188-94.
43. Taylor, A.W., J.W. Streilein, and S.W. Cousins, *Immunoreactive vasoactive intestinal peptide contributes to the immunosuppressive activity of normal aqueous humor.* Journal of Immunology, 1994. **153**(3): p. 1080-6.
44. Taylor, A.W., D.G. Yee, and J.W. Streilein, *Suppression of nitric oxide generated by inflammatory macrophages by calcitonin gene-related peptide in aqueous humor.* Investigative Ophthalmology and Visual Science, 1998. **39**(8): p. 1372-8.
45. Taylor, A.W. and D.G. Yee, *Somatostatin is an immunosuppressive factor in aqueous humor.* Investigative Ophthalmology and Visual Science, 2003. **44**(6): p. 2644-9.
46. Niederkorn, J.Y. and S. Wang, *Immune privilege of the eye and fetus: parallel universes?* Transplantation, 2005. **80**(9): p. 1139-44.
47. Griffith, T.S., et al., *CD95-induced apoptosis of lymphocytes in an immune privileged site induces immunological tolerance.* Immunity, 1996. **5**(1): p. 7-16.
48. Griffith, T.S., et al., *Fas ligand-induced apoptosis as a mechanism of immune privilege.* Science, 1995. **270**(5239): p. 1189-92.
49. Wang, S., et al., *Role of TRAIL and IFN-gamma in CD4+ T cell-dependent tumor rejection in the anterior chamber of the eye.* Journal of Immunology, 2003. **171**(6): p. 2789-96.
50. Sugita, S., et al., *Human corneal endothelial cells expressing programmed death-ligand 1 (PD-L1) suppress PD-1+ T helper 1 cells by a contact-dependent mechanism.* Investigative Ophthalmology and Visual Science, 2009. **50**(1): p. 263-72.
51. Osawa, H., K. Maruyama, and J.W. Streilein, *CD95 ligand expression on corneal epithelium and endothelium influences the fates of orthotopic and heterotopic corneal allografts in mice.* Investigative Ophthalmology and Visual Science, 2004. **45**(6): p. 1908-15.
52. Jerzak, M. and P. Bischof, *Apoptosis in the first trimester human placenta: the role in maintaining immune privilege at the maternal-foetal interface and in the trophoblast remodelling.* European Journal of Obstetrics, Gynecology, and Reproductive Biology, 2002. **100**(2): p. 138-42.
53. Choi, C. and E.N. Benveniste, *Fas ligand/Fas system in the brain: regulator of immune and apoptotic responses.* Brain Research. Brain Research Reviews, 2004. **44**(1): p. 65-81.
54. Shen, L., et al., *The function of donor versus recipient programmed death-ligand 1 in corneal allograft survival.* Journal of Immunology, 2007. **179**(6): p. 3672-9.
55. Hori, J., et al., *B7-H1-induced apoptosis as a mechanism of immune privilege of corneal allografts.* Journal of Immunology, 2006. **177**(9): p. 5928-35.
56. Prasse, A., et al., *Inhaled vasoactive intestinal peptide exerts immunoregulatory effects in sarcoidosis.* American Journal of Respiratory and Critical Care Medicine, 2010. **182**(4): p. 540-8.
57. Streilein, J.W., *Ocular immune privilege: the eye takes a dim but practical view of immunity and inflammation.* J Leukoc Biol, 2003. **74**(2): p. 179-85.

58. Wilbanks, G.A. and J.W. Streilein, *Distinctive humoral immune responses following anterior chamber and intravenous administration of soluble antigen. Evidence for active suppression of IgG2-secreting B lymphocytes.* Immunology, 1990. **71**(4): p. 566-72.
59. Streilein, J.W. and J.Y. Niederkorn, *Induction of anterior chamber-associated immune deviation requires an intact, functional spleen.* Journal of Experimental Medicine, 1981. **153**(5): p. 1058-67.
60. D'Orazio, T.J. and J.Y. Niederkorn, *Splenic B cells are required for tolerogenic antigen presentation in the induction of anterior chamber-associated immune deviation (ACAID).* Immunology, 1998. **95**(1): p. 47-55.
61. McKenna, K.C., Y. Xu, and J.A. Kapp, *Injection of soluble antigen into the anterior chamber of the eye induces expansion and functional unresponsiveness of antigen-specific CD8+ T cells.* Journal of Immunology, 2002. **169**(10): p. 5630-7.
62. McKenna, K.C. and J.A. Kapp, *Ocular immune privilege and CTL tolerance.* Immunologic Research, 2004. **29**(1-3): p. 103-12.
63. Niederkorn, J.Y. and J.W. Streilein, *Analysis of antibody production induced by allogeneic tumor cells inoculated into the anterior chamber of the eye.* Transplantation, 1982. **33**(6): p. 573-7.
64. Sonoda, K.H. and J. Stein-Streilein, *Ocular immune privilege and CD1d-reactive natural killer T cells.* Cornea, 2002. **21**(2 Suppl 1): p. S33-8.
65. Lin, H.H., et al., *The macrophage F4/80 receptor is required for the induction of antigen-specific efferent regulatory T cells in peripheral tolerance.* J Exp Med, 2005. **201**(10): p. 1615-25.
66. Taylor, A.W., *Ocular immune privilege.* Eye (Lond), 2009. **23**(10): p. 1885-9.
67. Sonoda, Y. and J.W. Streilein, *Impaired cell-mediated immunity in mice bearing healthy orthotopic corneal allografts.* J Immunol, 1993. **150**(5): p. 1727-34.
68. Niederkorn, J.Y., *Anterior chamber-associated immune deviation and its impact on corneal allograft survival.* Current Opinion in Organ Transplantation, 2006. **11**(4): p. 360-365.
69. She, S.C., L.P. Steahly, and E.J. Moticka, *Intracameral injection of allogeneic lymphocytes enhances corneal graft survival.* Investigative Ophthalmology and Visual Science, 1990. **31**(10): p. 1950-6.
70. Niederkorn, J.Y. and J. Mellon, *Anterior chamber-associated immune deviation promotes corneal allograft survival.* Investigative Ophthalmology and Visual Science, 1996. **37**(13): p. 2700-7.
71. Skelsey, M.E., J. Mellon, and J.Y. Niederkorn, *Gamma delta T cells are needed for ocular immune privilege and corneal graft survival.* Journal of Immunology, 2001. **166**(7): p. 4327-33.
72. Sonoda, Y. and J.W. Streilein, *Impaired cell-mediated immunity in mice bearing healthy orthotopic corneal allografts.* Journal of Immunology, 1993. **150**(5): p. 1727-34.
73. Yamagami, S. and M.R. Dana, *The critical role of lymph nodes in corneal alloimmunization and graft rejection.* Investigative Ophthalmology and Visual Science, 2001. **42**(6): p. 1293-8.
74. Williams, K.A., et al., *How effective is penetrating corneal transplantation? Factors influencing long-term outcome in multivariate analysis.* Transplantation, 2006. **81**(6): p. 896-901.

75. Coster, D.J. and K.A. Williams, *The impact of corneal allograft rejection on the long-term outcome of corneal transplantation.* American Journal of Ophthalmology, 2005. **140**(6): p. 1112-22.
76. Musch, D.C. and R.F. Meyer, *Risk of endothelial rejection after bilateral penetrating keratoplasty.* Ophthalmology, 1989. **96**(8): p. 1139-43.
77. Tuft, S.J., W.M. Gregory, and C.R. Davison, *Bilateral penetrating keratoplasty for keratoconus.* Ophthalmology, 1995. **102**(3): p. 462-8.
78. Maguire, M.G., et al., *Risk factors for corneal graft failure and rejection in the collaborative corneal transplantation studies. Collaborative Corneal Transplantation Studies Research Group.* Ophthalmology, 1994. **101**(9): p. 1536-47.
79. Kuchle, M., et al., *Risk factors for corneal allograft rejection: intermediate results of a prospective normal-risk keratoplasty study.* Graefes Archive for Clinical and Experimental Ophthalmology, 2002. **240**(7): p. 580-4.
80. Beauregard, C., et al., *Cutting edge: atopy promotes Th2 responses to alloantigens and increases the incidence and tempo of corneal allograft rejection.* J Immunol, 2005. **174**(11): p. 6577-81.
81. Price, M.O., R.W. Thompson, Jr., and F.W. Price, Jr., *Risk factors for various causes of failure in initial corneal grafts.* Archives of Ophthalmology, 2003. **121**(8): p. 1087-92.
82. Albuquerque, R.J., et al., *Alternatively spliced vascular endothelial growth factor receptor-2 is an essential endogenous inhibitor of lymphatic vessel growth.* Nature Medicine, 2009. **15**(9): p. 1023-30.
83. Cursiefen, C., et al., *Nonvascular VEGF receptor 3 expression by corneal epithelium maintains avascularity and vision.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(30): p. 11405-10.
84. Cursiefen, C., et al., *VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment.* Journal of Clinical Investigation, 2004. **113**(7): p. 1040-50.
85. Niederkorn, J.Y., et al., *Differential roles of CD8+ and CD8- T lymphocytes in corneal allograft rejection in 'high-risk' hosts.* Am J Transplant, 2006. **6**(4): p. 705-13.
86. Dietrich, T., et al., *Cutting edge: lymphatic vessels, not blood vessels, primarily mediate immune rejections after transplantation.* Journal of Immunology, 2010. **184**(2): p. 535-9.
87. Amescua, G., et al., *Effect of CXCL-1/KC production in high risk vascularized corneal allografts on T cell recruitment and graft rejection.* Transplantation, 2008. **85**(4): p. 615-25.
88. El-Sawy, T., M. Miura, and R. Fairchild, *Early T cell response to allografts occurring prior to alloantigen priming up-regulates innate-mediated inflammation and graft necrosis.* American Journal of Pathology, 2004. **165**(1): p. 147-57.
89. Fairchild, R.L., et al., *Expression of chemokine genes during rejection and long-term acceptance of cardiac allografts.* Transplantation, 1997. **63**(12): p. 1807-12.
90. Morita, K., et al., *Early chemokine cascades in murine cardiac grafts regulate T cell recruitment and progression of acute allograft rejection.* Journal of Immunology, 2001. **167**(5): p. 2979-84.
91. Steinman, R.M., *The dendritic cell system and its role in immunogenicity.* Annual Review of Immunology, 1991. **9**: p. 271-96.
92. Peeler, J.S. and J.Y. Niederkorn, *Antigen presentation by Langerhans cells in vivo: donor-derived Ia+ Langerhans cells are required for induction of delayed-type*

hypersensitivity but not for cytotoxic T lymphocyte responses to alloantigens. Journal of Immunology, 1986. **136**(12): p. 4362-71.
93. He, Y.G. and J.Y. Niederkorn, *Depletion of donor-derived Langerhans cells promotes corneal allograft survival.* Cornea, 1996. **15**(1): p. 82-9.
94. Ray-Keil, L. and J.W. Chandler, *Reduction in the incidence of rejection of heterotopic murine corneal transplants by pretreatment with ultraviolet radiation.* Transplantation, 1986. **42**(4): p. 403-6.
95. Ghoraishi, M., et al., *Penetrating keratoplasty in atopic keratoconjunctivitis.* Cornea, 1995. **14**(6): p. 610-3.
96. Hargrave, S., et al., *Preliminary findings in corneal allograft rejection in patients with keratoconus.* American Journal of Ophthalmology, 2003. **135**(4): p. 452-60.
97. Lyons, C.J., et al., *Sclerokeratitis after keratoplasty in atopy.* Ophthalmology, 1990. **97**(6): p. 729-33.
98. Niederkorn, J.Y., et al., *Allergic airway hyperreactivity increases the risk for corneal allograft rejection.* Am J Transplant, 2009. **9**(5): p. 1017-26.
99. Flynn, T.H., et al., *Effect of allergic conjunctival inflammation on the allogeneic response to donor cornea.* Investigative Ophthalmology and Visual Science, 2007. **48**(9): p. 4044-9.
100. Niederkorn, J.Y., *Immune regulatory mechanisms in allergic conjunctivitis: insights from mouse models.* Curr Opin Allergy Clin Immunol, 2008. **8**(5): p. 472-6.
101. Galatowicz, G., et al., *Ocular anti-allergic compounds selectively inhibit human mast cell cytokines in vitro and conjunctival cell infiltration in vivo.* Clin Exp Allergy, 2007. **37**(11): p. 1648-56.
102. Han, Y., J. Kim, and K. Ahn, *Food allergy.* Korean J Pediatr, 2012. **55**(5): p. 153-8.
103. Sampson, H.A., *Update on food allergy.* Journal of Allergy and Clinical Immunology, 2004. **113**(5): p. 805-19; quiz 820.
104. Takeda, K. and E.W. Gelfand, *Mouse models of allergic diseases.* Current Opinion in Immunology, 2009. **21**(6): p. 660-5.
105. Ozdoganoglu, T. and M. Songu, *The burden of allergic rhinitis and asthma.* Ther Adv Respir Dis, 2012. **6**(1): p. 11-23.
106. Akbari, O., *The role of iNKT cells in development of bronchial asthma: a translational approach from animal models to human.* Allergy, 2006. **61**(8): p. 962-8.
107. Wills-Karp, M., *Immunologic basis of antigen-induced airway hyperresponsiveness.* Annu Rev Immunol, 1999. **17**: p. 255-81.
108. Berend, N., C.M. Salome, and G.G. King, *Mechanisms of airway hyperresponsiveness in asthma.* Respirology, 2008. **13**(5): p. 624-31.
109. Bilkhu, P.S., J.S. Wolffsohn, and S.A. Naroo, *A review of non-pharmacological and pharmacological management of seasonal and perennial allergic conjunctivitis.* Cont Lens Anterior Eye, 2012. **35**(1): p. 9-16.
110. Bielory, L., *American Academy of Allergy, Asthma & Immunology--2008 Annual Meeting.* IDrugs, 2008. **11**(5): p. 309-11.
111. Chigbu, D.I., *The management of allergic eye diseases in primary eye care.* Cont Lens Anterior Eye, 2009. **32**(6): p. 260-72.
112. Bielory, L., *Ocular allergy overview.* Immunol Allergy Clin North Am, 2008. **28**(1): p. 1-23, v.

113. Bielory, L., *Allergic and immunologic disorders of the eye. Part II: ocular allergy.* Journal of Allergy and Clinical Immunology, 2000. **106**(6): p. 1019-32.
114. Nelms, K., et al., *The IL-4 receptor: signaling mechanisms and biologic functions.* Annual Review of Immunology, 1999. **17**: p. 701-38.
115. Scheinman, E.J. and O. Avni, *Transcriptional regulation of GATA3 in T helper cells by the integrated activities of transcription factors downstream of the interleukin-4 receptor and T cell receptor.* Journal of Biological Chemistry, 2009. **284**(5): p. 3037-48.
116. Li, Z., Y. Zhang, and B. Sun, *Current understanding of Th2 cell differentiation and function.* Protein Cell, 2011. **2**(8): p. 604-11.
117. Kim, S.H. and C.E. Lee, *Counter-regulation mechanism of IL-4 and IFN-alpha signal transduction through cytosolic retention of the pY-STAT6:pY-STAT2:p48 complex.* European Journal of Immunology, 2011. **41**(2): p. 461-72.
118. Hamelmann, E. and E.W. Gelfand, *IL-5-induced airway eosinophilia--the key to asthma?* Immunological Reviews, 2001. **179**: p. 182-91.
119. Akbari, O., et al., *Role of regulatory T cells in allergy and asthma.* Current Opinion in Immunology, 2003. **15**(6): p. 627-33.
120. Lisbonne, M., et al., *Cutting edge: invariant V alpha 14 NKT cells are required for allergen-induced airway inflammation and hyperreactivity in an experimental asthma model.* J Immunol, 2003. **171**(4): p. 1637-41.
121. Benlagha, K., et al., *A thymic precursor to the NK T cell lineage.* Science, 2002. **296**(5567): p. 553-5.
122. Pellicci, D.G., et al., *A natural killer T (NKT) cell developmental pathway iInvolving a thymus-dependent NK1.1(-)CD4(+) CD1d-dependent precursor stage.* Journal of Experimental Medicine, 2002. **195**(7): p. 835-44.
123. Ambrosino, E., J.A. Berzofsky, and M. Terabe, *Regulation of tumor immunity: the role of NKT cells.* Expert Opin Biol Ther, 2008. **8**(6): p. 725-34.
124. Seino, K. and M. Taniguchi, *Functionally distinct NKT cell subsets and subtypes.* J Exp Med, 2005. **202**(12): p. 1623-6.
125. Gober, M.D., et al., *Human natural killer T cells infiltrate into the skin at elicitation sites of allergic contact dermatitis.* Journal of Investigative Dermatology, 2008. **128**(6): p. 1460-9.
126. Bilenki, L., et al., *Natural killer T cells contribute to airway eosinophilic inflammation induced by ragweed through enhanced IL-4 and eotaxin production.* Eur J Immunol, 2004. **34**(2): p. 345-54.
127. Meyer, E.H., et al., *Glycolipid activation of invariant T cell receptor+ NK T cells is sufficient to induce airway hyperreactivity independent of conventional CD4+ T cells.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(8): p. 2782-7.
128. Akbari, O., et al., *CD4+ invariant T-cell-receptor+ natural killer T cells in bronchial asthma.* N Engl J Med, 2006. **354**(11): p. 1117-29.
129. Matangkasombut, P., et al., *Natural killer T cells in the lungs of patients with asthma.* Journal of Allergy and Clinical Immunology, 2009. **123**(5): p. 1181-5.
130. Sen, Y., et al., *V alpha 24-invariant NKT cells from patients with allergic asthma express CCR9 at high frequency and induce Th2 bias of CD3+ T cells upon CD226 engagement.* Journal of Immunology, 2005. **175**(8): p. 4914-26.

131. Mutalithas, K., et al., *Bronchoalveolar lavage invariant natural killer T cells are not increased in asthma.* Journal of Allergy and Clinical Immunology, 2007. **119**(5): p. 1274-6.
132. Rock, M., et al., *Effect of allergen challenge on the percentage of natural killer T cells in patients with atopic asthma.* Annals of Allergy, Asthma, and Immunology, 2009. **102**(5): p. 432-7.
133. Vijayanand, P., et al., *Invariant natural killer T cells in asthma and chronic obstructive pulmonary disease.* New England Journal of Medicine, 2007. **356**(14): p. 1410-22.
134. Scotet, E., et al., *Bridging innate and adaptive immunity through gammadelta T-dendritic cell crosstalk.* Frontiers in Bioscience, 2008. **13**: p. 6872-85.
135. Krug, N., et al., *Cytokine profile of bronchoalveolar lavage-derived CD4(+), CD8(+), and gammadelta T cells in people with asthma after segmental allergen challenge.* American Journal of Respiratory Cell and Molecular Biology, 2001. **25**(1): p. 125-31.
136. Lahn, M., et al., *Negative regulation of airway responsiveness that is dependent on gammadelta T cells and independent of alphabeta T cells.* Nature Medicine, 1999. **5**(10): p. 1150-6.
137. Zuany-Amorim, C., et al., *Requirement for gammadelta T cells in allergic airway inflammation.* Science, 1998. **280**(5367): p. 1265-7.
138. Isogai, S., et al., *Interferon-gamma-dependent inhibition of late allergic airway responses and eosinophilia by CD8+ gammadelta T cells.* Immunology, 2007. **122**(2): p. 230-8.
139. Hahn, Y.S., et al., *Different potentials of gamma delta T cell subsets in regulating airway responsiveness: V gamma 1+ cells, but not V gamma 4+ cells, promote airway hyperreactivity, Th2 cytokines, and airway inflammation.* Journal of Immunology, 2004. **172**(5): p. 2894-902.
140. Hahn, Y.S., et al., *V gamma 4+ gamma delta T cells regulate airway hyperreactivity to methacholine in ovalbumin-sensitized and challenged mice.* Journal of Immunology, 2003. **171**(6): p. 3170-8.
141. Hill, J.C., R. Maske, and P. Watson, *Corticosteroids in corneal graft rejection. Oral versus single pulse therapy.* Ophthalmology, 1991. **98**(3): p. 329-33.
142. George, A.J. and D.F. Larkin, *Corneal transplantation: the forgotten graft.* Am J Transplant, 2004. **4**(5): p. 678-85.
143. Sano, Y., J.W. Streilein, and B.R. Ksander, *Detection of minor alloantigen-specific cytotoxic T cells after rejection of murine orthotopic corneal allografts: evidence that graft antigens are recognized exclusively via the "indirect pathway".* Transplantation, 1999. **68**(7): p. 963-70.
144. Tanaka, K., K. Sonoda, and J.W. Streilein, *Acute rejection of orthotopic corneal xenografts in mice depends on CD4(+) T cells and self-antigen-presenting cells.* Investigative Ophthalmology and Visual Science, 2001. **42**(12): p. 2878-84.
145. Qian, Y. and M.R. Dana, *Molecular mechanisms of immunity in corneal allotransplantation and xenotransplantation.* Expert Rev Mol Med, 2001. **3**(18): p. 1-21.
146. Streilein, J.W., *Immunobiology and immunopathology of corneal transplantation.* Chemical Immunology, 1999. **73**: p. 186-206.
147. He, Y.G., J. Ross, and J.Y. Niederkorn, *Promotion of murine orthotopic corneal allograft survival by systemic administration of anti-CD4 monoclonal antibody.* Invest Ophthalmol Vis Sci, 1991. **32**(10): p. 2723-8.

148. Ayliffe, W., et al., *Prolongation of rat corneal graft survival by treatment with anti-CD4 monoclonal antibody.* Br J Ophthalmol, 1992. **76**(10): p. 602-6.
149. Niederkorn, J.Y., et al., *CD4+ T-cell-independent rejection of corneal allografts.* Transplantation, 2006. **81**(8): p. 1171-8.
150. Pepose, J.S., et al., *Composition of cellular infiltrates in rejected human corneal allografts.* Graefes Archive for Clinical and Experimental Ophthalmology, 1985. **222**(3): p. 128-33.
151. Torres, P.F., et al., *Cytokine mRNA expression during experimental corneal allograft rejection.* Experimental Eye Research, 1996. **63**(4): p. 453-61.
152. Yamada, J., J.W. Streilein, and M.R. Dana, *Role of tumor necrosis factor receptors TNFR-I (P55) and TNFR-II (P75) in corneal transplantation.* Transplantation, 1999. **68**(7): p. 944-9.
153. Niederkorn, J.Y., *The immunology of corneal transplantation.* Developments in Ophthalmology, 1999. **30**: p. 129-40.
154. Forstot, S.L. and H.E. Kaufman, *Corneal transplantation.* Annual Review of Medicine, 1977. **28**: p. 21-35.
155. Whitcup, S.M., et al., *Expression of cell adhesion molecules in corneal graft failure.* Cornea, 1993. **12**(6): p. 475-80.
156. Goldberg, M.F., T.A. Ferguson, and J.S. Pepose, *Detection of cellular adhesion molecules in inflamed human corneas.* Ophthalmology, 1994. **101**(1): p. 161-8.
157. He, Y., et al., *Effect of LFA-1 and ICAM-1 antibody treatment on murine corneal allograft survival.* Investigative Ophthalmology and Visual Science, 1994. **35**(8): p. 3218-25.
158. Hori, J., et al., *Specific immunosuppression of corneal allograft rejection by combination of anti-VLA-4 and anti-LFA-1 monoclonal antibodies in mice.* Experimental Eye Research, 1997. **65**(1): p. 89-98.
159. Yamagami, S., M. Isobe, and T. Tsuru, *Characterization of cytokine profiles in corneal allograft with anti-adhesion therapy.* Transplantation, 2000. **69**(8): p. 1655-9.
160. Cunnusamy, K., P.W. Chen, and J.Y. Niederkorn, *IL-17 promotes immune privilege of corneal allografts.* Journal of Immunology, 2010. **185**(8): p. 4651-8.
161. Coffman, R.L. and J. Carty, *A T cell activity that enhances polyclonal IgE production and its inhibition by interferon-gamma.* Journal of Immunology, 1986. **136**(3): p. 949-54.
162. Niederkorn, J.Y., et al., *Allergic conjunctivitis exacerbates corneal allograft rejection by activating Th1 and th2 alloimmune responses.* J Immunol, 2010. **184**(11): p. 6076-83.
163. Serody, J.S. and G.R. Hill, *The IL-17 differentiation pathway and its role in transplant outcome.* Biology of Blood and Marrow Transplantation, 2012. **18**(1 Suppl): p. S56-61.
164. Miossec, P., T. Korn, and V.K. Kuchroo, *Interleukin-17 and type 17 helper T cells.* New England Journal of Medicine, 2009. **361**(9): p. 888-98.
165. Luger, D., et al., *Either a Th17 or a Th1 effector response can drive autoimmunity: conditions of disease induction affect dominant effector category.* Journal of Experimental Medicine, 2008. **205**(4): p. 799-810.
166. Hirota, K., et al., *T cell self-reactivity forms a cytokine milieu for spontaneous development of IL-17+ Th cells that cause autoimmune arthritis.* Journal of Experimental Medicine, 2007. **204**(1): p. 41-7.
167. De Paiva, C.S., et al., *IL-17 disrupts corneal barrier following desiccating stress.* Mucosal Immunol, 2009. **2**(3): p. 243-53.

168. Yamada, J., et al., *MHC-matched corneal allograft rejection in an IFN-gamma/IL-17-independent manner in C57BL/6 mice.* Investigative Ophthalmology and Visual Science, 2009. **50**(5): p. 2139-46.
169. Cunnusamy, K., P.W. Chen, and J.Y. Niederkorn, *IL-17A-dependent CD4+CD25+ regulatory T cells promote immune privilege of corneal allografts.* Journal of Immunology, 2011. **186**(12): p. 6737-45.
170. Bluestone, J.A. and A.K. Abbas, *Natural versus adaptive regulatory T cells.* Nat Rev Immunol, 2003. **3**(3): p. 253-7.
171. Cunnusamy, K., et al., *Two different regulatory T cell populations that promote corneal allograft survival.* Investigative Ophthalmology and Visual Science, 2010. **51**(12): p. 6566-74.
172. Chauhan, S.K., et al., *Levels of Foxp3 in regulatory T cells reflect their functional status in transplantation.* J Immunol, 2009. **182**(1): p. 148-53.
173. Cunnusamy, K., P.W. Chen, and J.Y. Niederkorn, *Paradigm shifts in the role of CD4+ T cells in keratoplasty.* Discov Med, 2010. **10**(54): p. 452-61.
174. Cunnusamy, K., P.W. Chen, and J.Y. Niederkorn, *IL-17 Promotes Immune Privilege of Corneal Allografts.* J Immunol, 2010.
175. Shevach, E.M. and G.L. Stephens, *The GITR-GITRL interaction: co-stimulation or contrasuppression of regulatory activity?* Nat Rev Immunol, 2006. **6**(8): p. 613-8.
176. Fallarino, F., et al., *Modulation of tryptophan catabolism by regulatory T cells.* Nat Immunol, 2003. **4**(12): p. 1206-12.
177. Williams, K.A. and D.J. Coster, *The immunobiology of corneal transplantation.* Transplantation, 2007. **84**(7): p. 806-13.
178. Mottram, P.L., et al., *Increased expression of IL-4 and IL-10 and decreased expression of IL-2 and interferon-gamma in long-surviving mouse heart allografts after brief CD4-monoclonal antibody therapy.* Transplantation, 1995. **59**(4): p. 559-65.
179. Nickerson, P., et al., *Analysis of cytokine transcripts in pancreatic islet cell allografts during rejection and tolerance induction.* Transplantation Proceedings, 1993. **25**(1 Pt 2): p. 984-5.
180. Takeuchi, T., R.P. Lowry, and B. Konieczny, *Heart allografts in murine systems. The differential activation of Th2-like effector cells in peripheral tolerance.* Transplantation, 1992. **53**(6): p. 1281-94.
181. Klebe, S., et al., *Prolongation of sheep corneal allograft survival by transfer of the gene encoding ovine IL-12-p40 but not IL-4 to donor corneal endothelium.* Journal of Immunology, 2005. **175**(4): p. 2219-26.
182. Cui, J., et al., *Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors.* Science, 1997. **278**(5343): p. 1623-6.
183. Magone, M.T., et al., *A novel murine model of allergic conjunctivitis.* Clin Immunol Immunopathol, 1998. **87**(1): p. 75-84.
184. Leonardi, A., L. Motterle, and M. Bortolotti, *Allergy and the eye.* Clinical and Experimental Immunology, 2008. **153 Suppl 1**: p. 17-21.
185. Foster, C.S., *The pathophysiology of ocular allergy: current thinking.* Allergy, 1995. **50**(21 Suppl): p. 6-9; discussion 34-8.
186. Stern, M.E., et al., *Role of interferon-gamma in a mouse model of allergic conjunctivitis.* Invest Ophthalmol Vis Sci, 2005. **46**(9): p. 3239-46.

187. Ehlers, W.H. and P.C. Donshik, *Allergic ocular disorders: a spectrum of diseases.* CLAO Journal, 1992. **18**(2): p. 117-24.
188. Liu, G., et al., *Molecular and cellular aspects of allergic conjunctivitis.* Chem Immunol, 1999. **73**: p. 39-58.
189. Reyes, N.J., et al., *NKT cells are necessary for maximal expression of allergic conjunctivitis.* International Immunology, 2010. **22**(8): p. 627-36.
190. Ke, Y., et al., *Gamma delta T lymphocytes regulate the induction and maintenance of oral tolerance.* J Immunol, 1997. **158**(8): p. 3610-8.
191. Reyes, N.J., et al., *gammadelta T cells are required for maximal expression of allergic conjunctivitis.* Investigative Ophthalmology and Visual Science, 2011. **52**(5): p. 2211-6.
192. Fukushima, A., et al., *Modulation of murine experimental allergic conjunctivitis by treatment with alpha-galactosylceramide.* Immunol Lett, 2006. **107**(1): p. 32-40.
193. Ozaki, A., et al., *Phenotypic changes and inflammatory cell distribution in the cornea during development of experimental immune-mediated blepharoconjunctivitis.* Japanese Journal of Ophthalmology, 2004. **48**(4): p. 333-9.
194. Williams, K.A., et al., *Long-term outcome in corneal allotransplantation. The Australian Corneal Graft Registry.* Transplantation Proceedings, 1997. **29**(1-2): p. 983.
195. Pillemer, B.B., et al., *STAT6 activation confers upon T helper cells resistance to suppression by regulatory T cells.* Journal of Immunology, 2009. **183**(1): p. 155-63.
196. Liang, Q., et al., *IL-2 and IL-4 stimulate MEK1 expression and contribute to T cell resistance against suppression by TGF-beta and IL-10 in asthma.* Journal of Immunology, 2010. **185**(10): p. 5704-13.
197. Bielory, B.P., V.L. Perez, and L. Bielory, *Treatment of seasonal allergic conjunctivitis with ophthalmic corticosteroids: in search of the perfect ocular corticosteroids in the treatment of allergic conjunctivitis.* Curr Opin Allergy Clin Immunol, 2010. **10**(5): p. 469-77.
198. Thomas, J.K., et al., *The role of atopy in corneal graft survival in keratoconus.* Cornea, 2011. **30**(10): p. 1088-97.
199. Hauboldt, R. and S. Hanson, *2008 U.S. organ and tissue transplant cost estimates and discussion.* Milliman Research Report, 2008: p. 1-7.
200. Fukushima, A., et al., *Dissection of antigen-specific humoral and cellular immune responses for the development of experimental immune-mediated blepharoconjunctivitis in C57BL/6 mice.* Current Eye Research, 2005. **30**(4): p. 241-8.
201. Yokozeki, H., et al., *Gammadelta T cells assist alphabeta T cells in the adoptive transfer of contact hypersensitivity to para-phenylenediamine.* Clinical and Experimental Immunology, 2001. **125**(3): p. 351-9.
202. Brown, D.R., et al., *Beta 2-microglobulin-dependent NK1.1+ T cells are not essential for T helper cell 2 immune responses.* Journal of Experimental Medicine, 1996. **184**(4): p. 1295-304.
203. Korsgren, M., et al., *Natural killer cells determine development of allergen-induced eosinophilic airway inflammation in mice.* Journal of Experimental Medicine, 1999. **189**(3): p. 553-62.
204. Morishima, Y., et al., *Suppression of eosinophilic airway inflammation by treatment with alpha-galactosylceramide.* European Journal of Immunology, 2005. **35**(10): p. 2803-14.

205. Below, S., et al., *Virulence factors of Staphylococcus aureus induce Erk-MAP kinase activation and c-Fos expression in S9 and 16HBE14o- human airway epithelial cells.* Am J Physiol Lung Cell Mol Physiol, 2009. **296**(3): p. L470-9.
206. Hahn, Y.S., et al., *Vgamma1+ gammadelta T cells reduce IL-10-producing CD4+CD25+ T cells in the lung of ovalbumin-sensitized and challenged mice.* Immunology Letters, 2008. **121**(2): p. 87-92.
207. Reyes NJ, et al., *NKT cells are necessary for maximal expression of allergic conjunctivitis.* International Immunology, 2010. **In press**.
208. Jin, N., et al., *Airway hyperresponsiveness through synergy of gammadelta} T cells and NKT cells.* J Immunol, 2007. **179**(5): p. 2961-8.
209. Jin, N., et al., *Allergic airway hyperresponsiveness-enhancing gammadelta T cells develop in normal untreated mice and fail to produce IL-4/13, unlike Th2 and NKT cells.* J Immunol, 2009. **182**(4): p. 2002-10.
210. Chen, N. and E.H. Field, *Enhanced type 2 and diminished type 1 cytokines in neonatal tolerance.* Transplantation, 1995. **59**(7): p. 933-41.
211. Sayegh, M.H., et al., *CD28-B7 blockade after alloantigenic challenge in vivo inhibits Th1 cytokines but spares Th2.* Journal of Experimental Medicine, 1995. **181**(5): p. 1869-74.
212. Onodera, K., et al., *Distinct tolerance pathways in sensitized allograft recipients after selective blockade of activation signal 1 or signal 2.* Transplantation, 1999. **68**(2): p. 288-93.
213. Stumbles, P. and D. Mason, *Activation of CD4+ T cells in the presence of a nondepleting monoclonal antibody to CD4 induces a Th2-type response in vitro.* Journal of Experimental Medicine, 1995. **182**(1): p. 5-13.
214. Matesic, D., et al., *Type 2 immune deviation has differential effects on alloreactive CD4+ and CD8+ T cells.* Journal of Immunology, 1998. **161**(10): p. 5236-44.
215. Piccotti, J.R., et al., *IL-12 antagonism induces T helper 2 responses, yet exacerbates cardiac allograft rejection. Evidence against a dominant protective role for T helper 2 cytokines in alloimmunity.* Journal of Immunology, 1996. **157**(5): p. 1951-7.
216. VanBuskirk, A.M., M.E. Wakely, and C.G. Orosz, *Transfusion of polarized TH2-like cell populations into SCID mouse cardiac allograft recipients results in acute allograft rejection.* Transplantation, 1996. **62**(2): p. 229-38.
217. Gock, H., et al., *Cardiac and skin xenograft survival in different recipient mouse strains.* Transplantation, 2006. **82**(10): p. 1362-9.
218. Mohammad-Reza, A.B.-R.F.K.M.-A.J. and J.K.N.M.H.A. Anisian, *Corneal Graft Rejection: Incidence and Risk Factors.* Iran J Ophthalmic Res, 2007. **2**(1): p. 7-14.
219. Mosmann, T.R., et al., *Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins.* J Immunol, 1986. **136**(7): p. 2348-57.
220. Mosmann, T.R. and S. Sad, *The expanding universe of T-cell subsets: Th1, Th2 and more.* Immunol Today, 1996. **17**(3): p. 138-46.
221. Hargrave, S.L., J. Mellon, and J. Niederkorn, *MHC matching improves corneal allograft survival in mice with Th2-immune bias.* Transplantation Proceedings, 2002. **34**(8): p. 3413-5.
222. Ksander, B.R., Y. Sano, and J.W. Streilein, *Role of donor-specific cytotoxic T cells in rejection of corneal allografts in normal and high-risk eyes.* Transpl Immunol, 1996. **4**(1): p. 49-52.

223. Hegde, S. and J.Y. Niederkorn, *The role of cytotoxic T lymphocytes in corneal allograft rejection.* Invest Ophthalmol Vis Sci, 2000. **41**(11): p. 3341-7.
224. Pillemer, B.B., et al., *STAT6 activation confers upon T helper cells resistance to suppression by regulatory T cells.* J Immunol, 2009. **183**(1): p. 155-63.
225. Maerten, P., et al., *Effects of interleukin 4 on CD25+CD4+ regulatory T cell function.* Journal of Autoimmunity, 2005. **25**(2): p. 112-20.
226. Skapenko, A., et al., *The IL-4 receptor alpha-chain-binding cytokines, IL-4 and IL-13, induce forkhead box P3-expressing CD25+CD4+ regulatory T cells from CD25-CD4+ precursors.* Journal of Immunology, 2005. **175**(9): p. 6107-16.
227. Takaki, H., et al., *STAT6 Inhibits TGF-beta1-mediated Foxp3 induction through direct binding to the Foxp3 promoter, which is reverted by retinoic acid receptor.* Journal of Biological Chemistry, 2008. **283**(22): p. 14955-62.
228. Wei, J., et al., *Antagonistic nature of T helper 1/2 developmental programs in opposing peripheral induction of Foxp3+ regulatory T cells.* Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**(46): p. 18169-74.
229. Mantel, P.Y., et al., *GATA3-driven Th2 responses inhibit TGF-beta1-induced FOXP3 expression and the formation of regulatory T cells.* PLoS Biol, 2007. **5**(12): p. e329.
230. Cosmi, L., et al., *Th2 cells are less susceptible than Th1 cells to the suppressive activity of CD25+ regulatory thymocytes because of their responsiveness to different cytokines.* Blood, 2004. **103**(8): p. 3117-21.
231. Arnaboldi, P.M., F. Roth-Walter, and L. Mayer, *Suppression of Th1 and Th17, but not Th2, responses in a CD8(+) T cell-mediated model of oral tolerance.* Mucosal Immunol, 2009. **2**(5): p. 427-38.
232. Balic, A., et al., *IL-4R signaling is required to induce IL-10 for the establishment of T(h)2 dominance.* International Immunology, 2006. **18**(10): p. 1421-31.
233. Seder, R.A., et al., *The presence of interleukin 4 during in vitro priming determines the lymphokine-producing potential of CD4+ T cells from T cell receptor transgenic mice.* Journal of Experimental Medicine, 1992. **176**(4): p. 1091-8.
234. Paul, W.E., *Interleukin 4: signalling mechanisms and control of T cell differentiation.* Ciba Foundation Symposium, 1997. **204**: p. 208-16; discussion 216-9.
235. Zurawski, S.M., et al., *The primary binding subunit of the human interleukin-4 receptor is also a component of the interleukin-13 receptor.* Journal of Biological Chemistry, 1995. **270**(23): p. 13869-78.
236. Mattes, J., et al., *IL-13 induces airways hyperreactivity independently of the IL-4R alpha chain in the allergic lung.* Journal of Immunology, 2001. **167**(3): p. 1683-92.
237. Noben-Trauth, N., et al., *An interleukin 4 (IL-4)-independent pathway for CD4+ T cell IL-4 production is revealed in IL-4 receptor-deficient mice.* Proceedings of the National Academy of Sciences of the United States of America, 1997. **94**(20): p. 10838-43.
238. Noben-Trauth, N., J. Hu-Li, and W.E. Paul, *Conventional, naive CD4+ T cells provide an initial source of IL-4 during Th2 differentiation.* Journal of Immunology, 2000. **165**(7): p. 3620-5.
239. Roux, P.P. and J. Blenis, *ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions.* Microbiology and Molecular Biology Reviews, 2004. **68**(2): p. 320-44.

240. Jonuleit, H., et al., *Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood.* Journal of Experimental Medicine, 2001. **193**(11): p. 1285-94.
241. Annunziato, F., et al., *Phenotype, localization, and mechanism of suppression of CD4(+)CD25(+) human thymocytes.* Journal of Experimental Medicine, 2002. **196**(3): p. 379-87.
242. Takahashi, T., et al., *Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4.* Journal of Experimental Medicine, 2000. **192**(2): p. 303-10.
243. Tang, Q. and J.A. Bluestone, *The Foxp3+ regulatory T cell: a jack of all trades, master of regulation.* Nat Immunol, 2008. **9**(3): p. 239-44.
244. Read, S., et al., *Blockade of CTLA-4 on CD4+CD25+ regulatory T cells abrogates their function in vivo.* Journal of Immunology, 2006. **177**(7): p. 4376-83.
245. Niederkorn, J.Y., et al., *Allergic conjunctivitis exacerbates corneal allograft rejection by activating Th1 and th2 alloimmune responses.* Journal of Immunology, 2010. **184**(11): p. 6076-83.
246. Andersson, J., et al., *CD4+ FoxP3+ regulatory T cells confer infectious tolerance in a TGF-beta-dependent manner.* Journal of Experimental Medicine, 2008. **205**(9): p. 1975-81.
247. Duan, W., et al., *Anti-inflammatory effects of mitogen-activated protein kinase kinase inhibitor U0126 in an asthma mouse model.* Journal of Immunology, 2004. **172**(11): p. 7053-9.
248. Szczotka-Flynn, L., R. Ahmadian, and M. Diaz, *A re-evaluation of the risk of microbial keratitis from overnight contact lens wear compared with other life risks.* Eye Contact Lens, 2009. **35**(2): p. 69-75.
249. Dong, C., R.J. Davis, and R.A. Flavell, *MAP kinases in the immune response.* Annual Review of Immunology, 2002. **20**: p. 55-72.
250. Wong, W.S., *Inhibitors of the tyrosine kinase signaling cascade for asthma.* Curr Opin Pharmacol, 2005. **5**(3): p. 264-71.
251. Forward, N.A., et al., *Mast cells down-regulate CD4+CD25+ T regulatory cell suppressor function via histamine H1 receptor interaction.* Journal of Immunology, 2009. **183**(5): p. 3014-22.
252. Nguyen, K.D., C. Vanichsarn, and K.C. Nadeau, *Increased cytotoxicity of CD4+ invariant NKT cells against CD4+CD25hiCD127lo/- regulatory T cells in allergic asthma.* European Journal of Immunology, 2008. **38**(7): p. 2034-45.