DESTRUCTION OF CORNEAL NERVES PROMOTES
CORNEAL ALLOGRAFT REJECTION

APPROVED BY SUPERVISORY COMMITTEE

Jerry Y. Niederkorn, Ph.D.

Nancy Street, Ph.D.

Michelle Gill, M.D., Ph.D.

Rebecca Gruchalla, M.D., Ph.D.

DEDICATION

To my parents

Joseph and Nancy Paunicka

DESTRUCTION OF CORNEAL NERVES PROMOTES CORNEAL ALLOGRAFT REJECTION

by

Kathryn Joy Paunicka

Dissertation/Thesis Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

May, 2014

Copyright

by

KATHRYN JOY PAUNICKA, 2014

All Rights Reserved

ACKNOWLEDGEMENTS

DESTRUCTION OF CORNEAL NERVES PROMOTES

CORNEAL ALLOGRAFT REJECTION

KATHRYN JOY PAUNICKA

The University of Texas Southwestern Medical Center, 2014

Jerry Niederkorn, Ph.D.

The human corneal endothelium has very little regenerative capacities and cannot fully heal in response to infection or trauma. Evolutionarily, the eye developed a mechanism to retain visual acuity by protecting the eye from inflammatory damage, referred to as immune privilege. The mechanisms that protect the eye from inflammation-induced injury are 1.) the presence of immunosuppressive molecules within the aqueous humor; 2.) the expression of pro-apoptotic factors on corneal cells ; and 3.) the induction of a form of immune tolerance called anterior chamber-associated immune deviation (ACAID). Immune privilege contributes to the 90% success rate of corneal allografts without the need for histocompatibility matching and use of systemic immunosuppressive therapy.

However, when one or more parameters that contribute to immune privilege are broken, the cornel allograft becomes vulnerable to the immune system, resulting in corneal allograft failure. Patients that elect to have a corneal allograft replaced are the population at the highest risk of immune rejection and have only a 70% success rate with the second corneal allograft, and the rate of rejection continues to increase with each successive graft. Why subsequent corneal allografts have an increased incidence of rejection is unknown.

Due to the high success rate of corneal allografts, the donor tissues are not tissue matched to the recipients. With the limited documentation on the histocompatibility gene loci expressed by the corneal tissue donors, it is unknown if the rejection of the initial corneal allograft sensitizes the corneal allograft recipient. This study provides evidence that the sensitization of the corneal transplant recipient towards alloantigens also expressed on the subsequent corneal allografts is not a requisite for the high incidence of graft rejection. Furthermore, the enhanced incidence of graft rejection is an immune response directed towards alloantigens expressed on the subsequent corneal transplant.

The aim of this study is to determine why the corneal transplantation procedure enhances the rejection of subsequent corneal allografts in both eyes. Experimental evidence demonstrates that the destruction of the corneal nerves in one eye fundamentally alters the expression of the immunoregulatory neuropeptides in the contralateral eye. The altered expression of these neuropeptides inhibits both the induction and maintenance of immune privilege. The alteration in the microenvironment mediated a quick and prolonged loss of immune privilege, which could be reversed by blocking the activity of the pro-inflammatory neuropeptide substance P (SP).

The survival of the corneal allograft requires the participation of antigen-specific T regulatory cells. Neuropeptides within the ocular environment are important for immune

privilege through the induction of tolerance. Our results demonstrate the destruction of the corneal nerves and the release of the pro-inflammatory neuropeptide SP inhibits both the generation and function of T regulatory cells, which ultimately leads to corneal allograft rejection.

TABLE OF CONTENTS

PREVIOUS PUBLICATIONS

1. Cunnusamy, K.; Paunicka, K.; Reyes, N.; Yang, W.; Chen, P.W.; Niederkorn, J.Y., *Two different regulatory T cell populations that promote corneal allograft survival.* Investigative Ophthalmology and Visual Science, 2010. **51**(12): p. 6566-74.

2. Paunicka, K.; Chen, P.W.; Niederkorn J.Y., *Role of IFN-gamma in the establishment of anterior chamber-associated immune deviation (ACAID)-induced CD8+ T regulatory cells.* Journal of Leukocyte Biology, 2012. **91**(3): p. 475-83.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

6-OHDA	6-hydroxydopamine
ACAID	Anterior chamber-associated immune deviation
α-MSH	Alpha-melanocyte-stimulating hormone
AC	Anterior chamber
APC	Antigen presenting cell
AqH	Aqueous humor
BM	Bone marrow
CGRP	Calcitonin gene related peptide
CRP	Complement reactive peptide
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen -4
DC	Dendritic cell
DTH	Delayed type hypersensitivity
EIA	Enzyme immunoabsorbant assay
Foxp3	Forkhead box P3
GDNF	Glial cell line-derived neurotrophic factor
GITR	Glucocorticoid-induced tumor necrosis factor receptor family-related gene
GITRL	Glucocorticoid-induced tumor necrosis factor receptor family-related gene ligand
HLA	Human leukocyte antigen
HSK	Herpetic stromal keratitis
ICAM-1	Intercellular adhesion molecule 1

IDO	Indoleamine-2,3- dioxygenase
i.p.	Intraperitoneal
i.v.	Intravenously
LAP	Latent TGF-β-associated peptide
LASIK	Laser-assisted in situ keratomileusis
LAT	Local adoptive transfer assay
LC	Langerhans Cell
LFA-1	Lymphocyte function-associated antigen 1
MHC	Major histocompatibility complex
mH	Minor histocompatibility complex
MRT	Mean rejection time
MST	Median survival time
NGF	Nerve growth factor
NK-1R	Neurokinin receptor-1
NO	Nitric Oxide
NOS2	Nitric Oxide Synthase 2
PD-L1	program death ligand 1
PEDF	Pigment epithelial-derived factor
Pg.	Picogram
POMC	Proopiomelanocortin
ROS	Reactive oxygen species
SC	Subcutaneous
SP	Substance P

SO	Sympathetic ophthalmia
Sst	Somatostatin
TGF-β	Transforming growth factor beta
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
Treg	Regulatory T cell
TRPA1	Transient receptor potential ankyrin-repeat 1
TRPV1	Transient receptor potential vanilloid 1
VCAM	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VEGF-R1	Vascular endothelial growth factor receptor 1
VIP	Vasoactive intestinal peptide
VLA-4	Very late antigen-4
WT	Wild type

CHAPTER ONE

INTRODUCTION

Anatomy of the Cornea

The cornea is the most anterior tissue of the eye that provides the highest refractive index for vision and acts as a structural barrier against the external environment and infectious agents [1]. The cornea consists of five layers: three cellular layers (epithelium, stroma, and endothelium) and two interfacing layers (Bowman membrane and Descemet's membrane) [1].

Overlaying the epithelial surface is a mucinous tear film that provides immunological and growth factors. These factors maintain epithelial health by promoting cellular proliferation and repair. The tear film also protects the corneal surface from chemical, toxic, foreign-body, and microbial induced damage [1]. The epithelium is a stratified layer that is four to six cells thick (40 μm to 50 μm). The most superficial epithelial cells maintain tight junctions between each cell to prevent toxins and microbes from infiltrating into the deeper cellular layers of the cornea. The basal cell layer, the deepest cellular layer of the epithelium, is the only layer with mitotic activity and capable of regeneration. With the average epithelial cell lifespan of seven to ten days and the constant division, differentiation, and migration of stem cells from the limbus to the corneal surface, the total epithelial layer is completely turned-over in one week.

The stroma is the thickest portion of the cornea, comprising 85% of the corneal structure. The corneal stroma is transparent due to the parallel organization of the stromal fibers and extracellular matrix and the production of collagen and glycosaminoglycans by keratocytes [1]. In response to injury or infection, corneal stroma scaring can be persistent due to the migration of mobile keratocytes and the activation of fibroblasts [2]. Currently, it is only through surgical replacement of the corneal tissue that vision can be restored when stromal opacification occurs.

The endothelium is a 4 μm thick monolayer that covers the entire posterior portion of the cornea. Due to the lack of mitotic properties, the cellular density of the central endothelium decreases 0.6% cells per year, from 4,000 cells/mm^2 at birth down to 2,600 cells/mm^2 by the seventh decade of life. The endothelium's high density Na^+ K^+ ATPase ion pump system maintains the dehydration of the cornea by removing water from the stroma to the anterior chamber (AC). Corneas with an endothelial cell count below 500 cells/mm^2 due to age, trauma, or inflammation, are inefficient at maintaining a dehydrated stroma, become compromised and are at risk for developing corneal edema. The influx of water into the stroma disrupts the parallel organization of the fibers and results in corneal swelling and opacity.

The transparency of the cornea is also attributed to the absence of both lymph and blood vessels. The avascularity of the cornea is due to the presence of anti-hemangiogenic factors thrombospondin-1, vascular endothelial growth factor receptor 1 (VEGF-R1), pigment endothelial-derived factor (PEDF), endostatin, and angiostatin within the cornea [3]. VEGF-R1 prevents angiogenesis by sequestering Flt and inhibits it from binding to vascular endothelial growth factor (VEGF). Thrombospondin-1 activates CD36 on macrophages and endothelial cells and prevents the secretion of VEGF [4]. Angiostatin and endostatin promote cell cycle arrest and apoptosis of vascular endothelial cells [5].

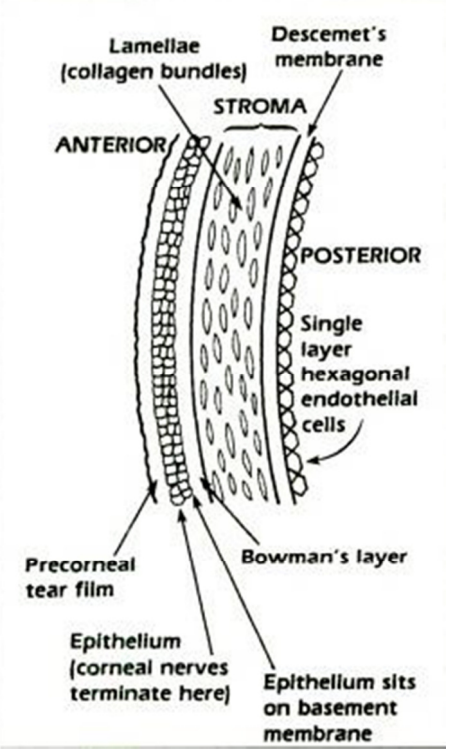


Figure 1. Anatomical structure of the cornea. The cornea is a cellular structure at the anterior portion of the eye. The epithelium is a cellular barrier that prevents the infiltration of environmental pathogens. The stroma is a thick transparent stratified collagenous layer. The endothelial layer dehydrates the cornea through ionic pumps. Image adapted from Ocular Anatomy and Physiology [6]

The cornea is one of the most densely innervated tissues within the human body with approximately 7,000 nerve endings/mm^2 [7]. The rich supply of sensory and autonomic sensory nerve fibers is involved in the transmission of pain and noxious stimuli. In humans, the nerves bundles from the ophthalmic branch of the trigeminal nerve enter into the cornea parallel to the ocular surface in a radial fashion. The unmyelinated axon nerve trunks subdivide into several branches and travel through the anterior third of the stroma towards the central cornea. The stromal nerve fibers turn 90^o towards the ocular surface and penetrate into the Bowman's layer. After entering into the Bowman's layer, the nerves continue to divide and turn 90^o to run parallel to the ocular surface to form the nerve plexus. The nerve fibers branch off from the subbasal nerve plexus and project anteriorly through the epithelium and terminate at the corneal surface [7]. The presence of the corneal nerves, and the factors they produce, regulate corneal sensation, maintain a healthy cornea epithelium, and promote wound healing after injury [8, 9]. The release of neurotransmitters and neuropeptides promotes type VII collagen production, epithelial cell growth, proliferation, differentiation, and migration [8, 10]. In turn, the release of neurotrophins, nerve growth factor (NGF), and glial cell line-derived neurotrophic factor (GDNF) by epithelial cells and stromal keratocytes promote nerve growth and survival within the cornea [11-14].

Patients with dry eye [15], herpetic ocular infection [16], stroke, diabetes [17], corneal surgery [18, 19], or cranial lesions [20] have a disruption in the corneal innervation processes that results in neurotrophic keratopathy. Without the neuropeptides supplied by the nerves the cornea develops ulcerations and perforations resulting in the desiccation of the corneal surface [7]

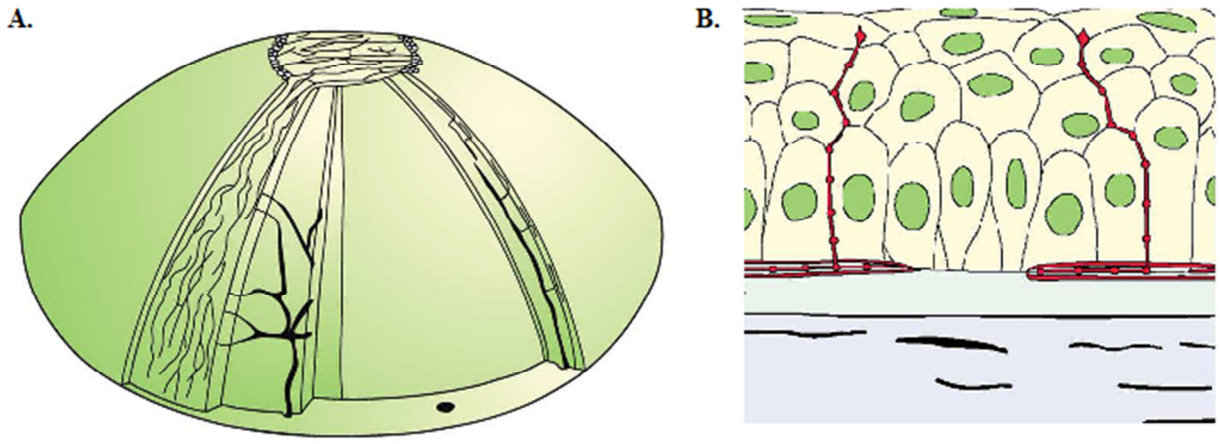


Figure 2. Innervation of the corneal nerves. A.) The radial innervation distribution of large nerve fibre bundles and the fine nerve endings within the stroma and subbasal plexus, respectively. B.) The architecture of nerves in the subbasal plexus that bifurcate and turn upwards into the epithelium. Adapted from Müller et. al. Exp. Eye Res. 2003.

History of Corneal Transplantation

When a cornea becomes cloudy, light cannot penetrate the eye to reach the light-sensitive retina and results in poor vision and even blindness. In 1796, Erasmus Darwin suggested the concept of replacing an opaque cornea as a therapy to restore vision [21]. It was not until 1835 when Samuel Bigger successfully transplanted an allogeneic cornea onto the blind eye of a gazelle did replacement of the corneal tissue have a practical clinical application [21]. In 1838, Richard Kissans transplanted a pig xenograft onto a blind human as an attempt to use corneal transplantation as a therapeutic application to alleviate blindness. The graft succeeded for a few weeks before increasing in opacity [21]. It was not until 1905 that the first successful corneal transplant on humans was performed by Austrian ophthalmologist Eduard Konrad Zirm [22]. Since that time, corneal transplants have become one of the most common solid tissue transplants with over 40,000 transplantations performed in the US each year, surpassing the number of heart, liver and kidney transplants [23]. The reasons for corneal transplantation are summarized in Table 1.

Reason for Corneal Transplantation	Number of corneal transplants
Keratoconus	6,650 (18.1%)
Repeat corneal transplant	4,460 (12.1%)
Post-refractive surgery	80 (0.2%)
Endothelial Dystrophies (Fuchs')	1,400 (3.8%)
Microbial changes	907 (2.5%)
Mechanical or chemical trauma	1,268 (3.5%)
Congenital opacities	651 (1.8%)
Pterygium	15 (0.004%)
Non-infectious ulcerative keratitis	1,211 (3.2%)
Other corneal dysfunction or distortion (non-endothelial)	3,795 (10.3%)
Other degenerations or dystrophies	1,713 (4.7%)
Other Endothelial Dysfunction	1,131 (3.1%)
Unknown, Unreported, or Unspecified	9,765 (26.6%)
Total number of corneal transplants	36,716

Table 1. Total Number of Corneal Transplants Performed in the U.S. In 2012, the most common known specified reasons for corneal transplantation were keratoconus (4,460, 18.1%), followed by repeat corneal transplantation (3,670, 12.1%).

Corneal transplantation has a high acceptance rate; approximately 74% graft survival over 5 years [24]. This is a remarkable success rate considering the conditions in which corneal transplantation is conducted. Unlike kidney or heart transplantation, which require the use of systemic immunosuppressive treatment over the lifespan of the patient, corneal transplants rely only on the use of topical corticosteroid treatment [25]. Moreover, unlike kidney and heart transplantation, where histocompatibility matching between the donor and recipient are crucial for graft survival, histocompatibility matching is not required for corneal transplants. Rather, it has been suggested that ABO blood group compatibility has a greater impact than major histocompatibility complex (MHC) matching on the survival of the corneal allograft [25].

In rodent models, where both the major histocompatibility and minor histocompatibility markers expressed on the donor cornea are mismatched from the recipient, 50% of the corneal allografts are accepted [26, 27]. This is a stark contrast to skin allografts, which under similar conditions, undergo 100% rejection. The high success rate of corneal allografts under low stringent conditions is dependent on maintaining all of the factors that support immune privilege.

Ocular Immune Privilege

Immune privilege is the evolutionary adaptation that induces immune tolerance, and thus protects a particular tissue or site from the damaging effects from an inflammatory immune response towards non-self-antigens. Immune privilege is a tissue-specific feature of ocular tissue, brain, testes, pregnant female uterus, and hair follicle. The first recorded observation of the immune privilege status of the anterior chamber (AC) of the eye was made by van Dooremal, who noted that a mouse skin graft had prolong survival when it was placed into the AC of a dog. Sir Peter Medawar observed that grafts placed in the skin underwent rapid rejection, while implantation of the same tissue into the AC or brain had prolonged survival since they were not attacked by the immune system [28]. He postulated that the avascularity and absence of lymphatic drainage of the eye and the brain prevented the graft from recognition by the immune system, a theory known as 'immunological ignorance'. However, further investigations demonstrated that the immune privilege of the eye is a cell-mediated regulatory mechanism that prevents an immune reaction from mediating an inflammatory response [29-31]. Kaplan and Streilein showed that injection of allogeneic lymphoid cells into the AC of the eye altered the systemic immune response, which resulted in high levels of alloantibodies; yet cellular responses were suppressed and resulted in the reduced rejection of orthotopic skin grafts expressing the same alloantigens as those expressed on the lymphoid cells injected into the AC. This form of immune regulation, referred to as anterior chamber-associated immune deviation (ACAID), is believed to support the immune privilege of the eye and maintain corneal allograft survival [32, 33].

Corneal transplants overlay the aqueous humor (AqH) and are in direct contact with the numerous immunuosuppressive and anti-inflammatory molecules. The corneal allograft

endothelial cells sloughed off into AC are analogous to an AC injection of alloantigens and induce features similar to ACAID. Recipients with long-term surviving corneal allografts down-regulate delayed type hypersensitivity (DTH) responses to the alloantigens expressed on the corneal allograft [34]. Additionally, manipulations that prevent the induction of ACAID also deprive the corneal allograft of its immune privilege and result in allograft rejection [35, 36]. Splenectomy and the depletion of natural killer T cells inhibits the induction of ACAID and also results in increased corneal allograft rejection [37, 38]. Moreover, the induction of ACAID by injecting allogeneic cells into the AC promotes corneal allograft survival [38].

Many of the anatomical, physiological, and immunoregulatory factors that contribute to the induction of ACAID are also required for the success of corneal allograft survival. Without the function of each of these factors, the eye becomes vulnerable to immune-mediated inflammatory damage which results in a loss of vision. How each of the factors contribute to immune privilege and promote corneal allograft survival are outlined below.

Avascularity of the Cornea

The cornea is an avascular tissue lacking both blood vessels and lymphatic vessels. This avascularity is due to the presence of the anti-hemangiogenic and anti-lymphangiogenic factor VEGFR3 [39]. The absence of lymphatic vessels to connect the cornea to regional lymph nodes results in limited emigration of Langerhans cells (LC) out of the cornea and reduces corneal allograft rejection [40]. Unlike LCs contained within the skin, LCs contained within the cornea has limited expression of MHC II molecules. The reduction of MHC class II expression limits antigen presenting cells (APC) from presenting foreign antigens to lymphocytes, which in turn reduces the potency of corneal LCs to initiate the inflammatory response. Thus, the limitation of

MHC class II^+ cells within the cornea prevents sensitization of the immune system toward foreign MHC class II and foreign antigens via direct and indirect pathways of presentation, respectively [41].

Soluble Factors

The AqH that fills the anterior chamber of the eye is a clear fluid that contains an array of immunoregulatory factors that modulate the immune response (Table 2). The AqH from normal eyes contains immunosuppressive molecules that influence the differentiation and activation of macrophages, inhibit T cell activation, and convert T cells to regulatory T cells (Tregs) [42]. The AqH alters infiltrating T cells by inhibiting IFN-γ production by Th1 cells and converts the $CD4^+$ T cells towards having a suppressive function. The AqH also contains high levels of transforming growth factor beta (TGF-β), a potent immunosuppressant. APCs exposed to TGF-β *in vitro* and injected intravenously (i.v.) into naïve mice induce ACAID [43]. Indoleamine dioxygenase (IDO) is an enzyme that catabolizes the amino acid tryptophan needed for T cell survival [44]. The expression of IDO within the cornea is important for maintaining immune privilege by limiting $CD4^+$ T cell proliferative responses and promoting Treg generation [45].

Many of the soluble molecules associated with immune privilege are neuropeptides, which implies a direct link between the nervous system and immune responses in the ocular environment. Accumulating evidence suggests a critical role of the nervous system in regulating immunological reactions. The crosstalk between the nervous and immune systems is mediated by the soluble products, such as neuropeptides and cytokines, which interact with receptors present on both immune and nervous system cells.

The neuropeptide α-MSH is a 13-amino acid-long byproduct from the cleavage of pro-opiomelanocortin hormone. Within the AqH, α-MSH is constitutively expressed and maintains the immunosuppressive environment of the eye by acting at both the innate and adaptive arms of the immune response. α-MSH blocks TLR-signaling pathways in macrophages and inhibits activation of NF-κB and p38 MAPK. APC's under the influence of α-MSH in the AqH emigrate to the spleen to produce TGF-β and IL-10 and promote the expansion of Tregs [46]. Locally, α-MSH does not prevent the proliferation of activated T cells, rather it modifies activated infiltrating $CD4^+$ T cells from IFN-γ-producing Th1 cells and converts them to TGF-β-producing Tregs [47]. The ability of α-MSH to induce Tregs within the eye is obstructed when melanocortin 5 receptor is blocked [48].

Vasoactive intestinal peptide (VIP) is a 28 amino acid anti-inflammatory neuropeptide that has been detected in corneal nerves and AqH. VIP protects the cornea from perforation during *Pseudomonas aeruginosa* keratitis by facilitating wound healing and epithelial barrier function by regulating growth factors EGF, FGF, HGF and VEGF-A; adhesion molecules vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1); and proinflammatory mediators nitric oxide (NO), TNF-α, IFN-γ, and IL-1β; and by promoting anti-inflammatory cytokines TGF-β, and IL-10 during infection [49-51]. VIP provides a protective effect during endotoxemia by inhibiting macrophage and T cell proliferation and by the reducing IL-6 and TNF-α production [52, 53].

Substance P (SP) and calcitonin gene-related peptide (CGRP) are neuropeptides that are co-localized within the sensory corneal nerves and are secreted into the corneal tissue and AqH [54, 55]. Both SP and CGRP have been implicated in promoting corneal wound healing

responses and in immunomodulatory responses. The presence of CGRP in the AqH prevents inflammation by limiting nitric oxide synthase 2 (NOS2) enzymatic activities and limits the production of NO by macrophages [56].

The neuropeptide SP is an 11 amino acid long member of the tachykinin family. SP and its receptor, neurokinin receptor-1 (NK-1R), are present in the local ocular environment in normal corneal nerves, epithelium, and tears [57, 58] as well as in peripheral leukocytes [59-61]. SP is important for wound healing and regenerating the corneal epithelium by stimulating epithelial cell proliferation, migration and adhesion [62]. SP promotes epithelial cell-to-cell adhesion and adhesion of epithelial cells to fibronectin by upregulating adhesion molecules and cytoskeleton proteins, such as E-cadherin and alpha 5 integrin [63-65]. Moreover, SP has been implicated in protecting the ocular environment from outside pathogens by promoting inflammation at both the innate and adaptive arms of the immune reflex arc. SP promotes vasodilatation and angiogenesis within the cornea by stimulating endothelial cell proliferation and activation of NO synthase [66]. In conjunction with cytokine signaling SP can affect B cell function by driving isotype switching of antibodies [67]. However, the protective effects of SP against invading pathogens may have deleterious effects on immune privilege by inhibiting the induction of ACAID. Upregulating the expression of SP and its receptor in the ocular environment prevents the induction of ACAID and the suppression of DTH responses [68-70].

Molecule	Effect on the Immune Response
TGF-β	Inhibits T cell activation, proliferation, and effector function Alters APC function
VIP	Inhibits T cell activation
α-MSH	Promotes APC to induce tolerance
IDO	Prevents T cell proliferation
CRP	Prevents Complement Activation
CGRP	Inhibits Macrophage production of NO
SP	Promotes T cell activation, prevents Treg Generation

Table 2. Soluble Immunosuppressive Molecules Contained within the AqH. TGF-β, transforming growth factor Beta; VIP, vasoactive intestinal peptide; α-MSH, alpha melanocyte-stimulating hormone; IDO, indoleamine dioxygenase; CRP, complement reactive peptide; CGRP, calcitonin gene related peptide; SP, substance P.

Cell Membrane Bound Molecules

The immune privilege of the eye is also maintained by cell membrane-bound molecules expressed on the endothelial cell layer (Table 3). The pigmented epithelium of the iris and ciliary body and the corneal endothelium, express TGF-β, glucocorticoid-induced tumor necrosis factor receptor family-related gene ligand (GITRL), FasL, program death ligand 1 (PD-L1), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).

GITRL and the membrane bound form of TGF-β2 are constitutively expressed on the corneal endothelium. The ligation of GITRL to glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR) converts $CD4^+$ T effector cells to $CD4^+CD25^+$ Tregs and induces the expansion of forkhead box P3 (FoxP3) expressing cells [71]. GITR prevents the efferent arm of the immune reflex arc by inducing tolerance and preventing the infiltration of T effector cells into the cornea, thus protecting the cornea from T cell-mediated destruction [71]. Moreover, the interaction of $CD8^+$ T cells with membrane bound TGF-β on human corneal endothelial cells converts them to FoxP3 expressing cells [72]. The converted $CD8^+FoxP3^+$ Tregs mediate the suppression of T effector cells by secreting the soluble form of TGF-β1 [72]. The expression of these molecules blocks inflammatory cell-mediated graft rejection by converting effector T cells to regulatory $FoxP3^+$ Tregs, thus effectively contributing to immune privilege through the induction of localized tolerance.

FasL, TRAIL, and PD-L1 are molecules expressed on the corneal endothelium and epithelium [73-75]. The expression of these factors is a tactical way for the cornea to induce apoptotic cell death of inflammatory cells, such as T cells and macrophages, thus blunting the infiltration of inflammatory cells and inhibiting the destruction of the corneal graft [74-80]. B6

corneal allografts, which do not expression functional FasL (gld/gld), transplanted to BALB/c recipients have a higher incidence of graft rejection compared to their wild type (WT) counterparts [81, 82]. The heightened incidence of graft rejection is attributed to the reduced capacity of corneal allografts to induce apoptosis of Fas^+ inflammatory cells at the graft/host interface [81, 82]. The expression of PD-L1 is also essential for the immune privilege of corneal allografts, as PD-L1 deficient corneal allografts transplanted onto BALB/c recipients have a high incidence of graft rejection [75, 83].

Molecule	Immune Response Effect
FasL	Promotes T cell apoptosis
GITRL	Promotes Treg proliferation
mTGF-β	Suppresses T cell, macrophage activation
PD-L1	Induces T cell apoptosis
TRAIL	Induces macrophage apoptosis

Table 3. Membrane-Bound Suppressive Molecules Expressed on the Cornea. FasL, Fas ligand; GITRL, glucocorticoid-induced tumor necrosis factor receptor family-related gene ligand; mTGF-β, Membrane bound transforming growth factor-beta; PD-L1, programmed cell death ligand -1; TRAIL, Tumor necrosis factor related-apoptosis inducing ligand.

Anterior Chamber-Associated Immune Deviation

Seminal studies by Niederkorn and Streilein demonstrated that antigens injected into the AC induce an antigen specific tolerogenic immune response [84]. This deviant immune response termed ACAID, is characterized by the suppression of Th1 immune responses, the deviation of antibody production towards non-complement fixing murine IgG1 antibodies, and the generation of $CD4^+CD25^+$ and $CD8^+$ Treg [85, 86]. The injection of viral proteins, soluble proteins, or allogeneic cells into the AC, induces ACAID [85, 87, 88]. Within 48 hours of AC injection of antigen, residential $F4/80^+$ APCs migrate from the iris/ciliary body and enter the TGF-β–rich AqH to capture the injected antigen. These tolerance inducing, IL-10-secreting APCs migrate out of the ocular environment through the canal of Schlemm and enter the venous drainage of the eye and migrate to the thymus [89]. In the thymus, the APCs induce the generation of $CD4^-CD8^-$ $NK1.1^+$ thymocytes. These APCs and thymocytes then travel to the spleen, where they interact with B cells, NKT cells and γδ T cells [88, 90, 91]. The eye, thymus, and the spleen are all highly innervated tissues that are supplied by the sympathetic nervous system and coincidentally, are crucial for the induction of ACAID [7, 92]. Chemical sympathectomy of the peripheral nervous system with the administration of 6-hydroxydopamine (6-OHDA) prevents the induction of immunoregulatory thymic NK 1.1^+ cells and $CD8^+$ Tregs [93]. Without the presence of an intact and fully innervated spleen and thymus, ACAID cannot be induced and the generation of $CD4^+CD25^+$ afferent Tregs and $CD8^+$ efferent Tregs is abrogated [94-96].

There is evidence suggesting that the induction of ACAID supports the survival of corneal transplants [38]. Similar to the direct injection of antigens within the eye, the shedding of alloantigens from a transplanted corneal graft into the AC can induce a suppressive response [97]. Similar to ACAID, recipients that maintained a clear graft could suppress an antigen-

specific DTH responses to alloantigens expressed on the corneal transplants [34, 98]. Also, inducing ACAID by injecting donor alloantigens into the eye prior to transplantation induces alloantigen specific tolerance and promotes corneal allograft survival [38, 99]. Likewise, blocking the induction of ACAID, leads to increased corneal allograft rejection [35, 100].

Mechanism of Corneal Allograft Rejection

Immune rejection remains the leading cause for corneal allograft failure [101]. All three layers of the cornea are susceptible to immune-mediated attack. The epithelial layer has a rapid turnover, is easily repaired within a matter of weeks, and has no significant effect on the survival of the graft. The endothelial cell layer is required for the deturgescence of the stromal layer and immune-mediated attack leads to the swelling of the stromal layer, disruption of the collagen fiber orientation, and the development of stromal edema, which ultimately results in the opacification of the corneal tissue. Damage to the endothelial cell layer is irreversible due to the lack of mitotic activity of the corneal endothelial cells and their inability to regenerate a functional endothelium. Thus, the structural and functional integrity of the endothelial cell layer is crucial for the survival of the corneal allograft.

Human studies on the pathogenesis of corneal transplantation have been limited. The establishment of the rodent model of corneal transplantation by Williams and Coster has advanced the understanding of the processes involved in corneal allograft rejection [102]. The first phase of corneal transplantation rejection is the induction of the immune response through the presentation of alloantigen by APCs. There are two distinct pathways of allorecognition; the presentation of donor MHC molecules on the surface of APCs in the direct pathway, and the presentation of processed donor MHC or mH by host APCs in the indirect pathway [103]. After the cornea is transplanted, regional APCs become activated and emigrate out of the eye and travel to the draining lymph node. The second phase is the sensitization of the adaptive immune response towards the corneal alloantigen. The APCs present the alloantigen to the host's $CD4^+$ T cell's T cell receptor. The $CD4^+$ T cells become activated and migrate out of the lymphoid

organs back to the corneal tissue and mediate their destructive functions against the allograft [104].

However, it should be noted that the mechanism of corneal allograft rejection in the mouse may not fully represent the mechanism of rejection in the human. Yet, the advancements made in the murine model of corneal transplantation provide the greatest asset in understanding how corneal allografts undergo rejection, which in turn assists in designing effective future therapies. Currently, the primary therapy for preventing immune-mediated assault of a corneal allograft is the topical application of glucocorticoids to the transplanted tissue [105].

Role of T Lymphocytes in Corneal Allograft Rejection

The rejection of skin and kidney allogeneic transplants is mediated by the collaborative destructive functions of $CD4^+$ T cells and $CD8^+$ T cells (CTLs) [106, 107]. Furthermore, substantial evidence suggests that corneal transplantation is a T cell-mediated process [108, 109]. However, the induction of CTLs is not required for corneal allograft rejection. Previous studies demonstrated that CTL cytotoxic responses against the cornea are not elicited in low risk corneal allograft recipients [110, 111]. Moreover, $CD8^+$ T cell knockout mice were as effective as WT mice in the rejection of a corneal allograft [111, 112]; suggesting that CTLs are not required for corneal allograft rejection. However, CTLs from prevascularized hosts or from $CD4^{-/-}$ corneal allograft rejectors are generated and can independently mediate graft rejection [113]. *In vitro* assays suggest the CTL-mediated graft rejection is not due to direct cytolysis of the corneal endothelium; instead, destruction of the corneal allograft is a DTH-mediated response [111, 114].

Clinical investigations suggest that antibodies may also contribute to corneal allograft rejection [115-117]. Corneal transplant recipients that rejected their allografts have elevated level of alloantibodies in their serum, suggesting that B cells could have a role in the rejection process. Investigations that used the mouse model of penetrating keratoplasty to examine if B cells and the antibodies they produce can independently mediate graft rejection have produced mixed results. Forrester et. al. noted that the passive transfer of serum from BALB/c mice subcutaneously sensitized towards B10 alloantigens into BALB/c corneal allograft recipients resulted in the increased incidence of graft rejection [118]. However, the transfer of serum from B6 immunized BALB/c mice into B cell-deficient nude BALB/c recipients resulted in increased opacity of B6 corneal allografts through a complement dependent mechanism but did not result in heightened corneal allograft rejection [119]. Moreover, B cell-deficient mice have the same incidence of graft rejection as WT recipients [120]. These results suggest that B cells and antibodies alone are not sufficient in mediating corneal allograft rejection but can contribute to the destruction of corneal allografts.

Multiple studies demonstrated that $CD4^+$ T cells are vitally important in corneal allograft rejection [112, 121-124]. Investigations in rodents revealed that *in vivo* depletion of $CD4^+$ T cells significantly reduces the incidence of immune rejection of corneal allografts [123, 125, 126]. Moreover, adoptive transfer experiments demonstrated that $CD4^+$ T cells induced an alloantigen-specific immune response and led to corneal allograft rejection. $CD4^+$ T cells have various functionally unique subsets, including Th1, Th2, Th17, and Tregs, and are defined by the unique cytokines and chemokines they produce. The unique functions of the $CD4^+$ T cell subsets promote DTH inflammation or tolerance and result in the rejection or survival of corneal allografts.

Role of Th1 cells in Corneal Allograft Rejection

Th1 $CD4^+$ T cells are characterized by their production of IFN-γ and expression of the transcription factor T-bet. Th1 cells stimulate macrophages and endothelial cells to produce inflammatory factors, primarily against intracellular bacteria, and viruses, but can also be linked to autoinflammatory responses, such as arthritis [127-130]. The presence of $CD4^+$ T cells within rejected corneal allografts led to the notion that Th1 cells are the primary mediators of corneal allograft rejection by activating DTH [34, 109, 131, 132]. It has been demonstrated that the Th1-associated cytokines, IFN-γ and TNF-α, protein and mRNA levels are elevated in rejected corneal allografts [132, 133]. Th1 cytokines act directly on the corneal tissue and mediate graft rejection by promoting the production of NO, which activates apoptosis of the corneal endothelial cells [134]. Blocking iNOS, either through transcriptional and translational regulation by the inhibitor FK506 or at the enzymatic level with aminoguanidine, results in heightened corneal allograft acceptance [135]. Th1 cytokines mediate the recruitment of macrophages to the graft through the upregulation of adhesion molecules VCAM-1, ICAM-1, and E-selectin [136, 137]. Preventing the adhesion of ICAM-1 and VCAM-1 to their integrins lymphocyte function-associated antigen 1 (LFA-1) and very late antigen-4 (VLA-4) respectively, reduced DTH responses and promoted corneal allograft acceptance [138, 139]. However, the exact role of IFN-γ in corneal allograft rejection is not completely understood. Some studies have shown inactivation of IFN-γ results in exacerbated rejection of corneal allografts mismatched with the host at the entire MHC plus all known minor histocompatibility (mH) gene loci [140, 141]. The IFN-γ–deficient recipients produced a Th2 immune response that resulted in the infiltration of eosinophils and exacerbation of allograft rejection [142]. By contrast, MHC-matched corneal allografts that express only mH alloantigens enjoy long term survival in

over 90% of IFN-γ-deficient hosts [143]. This demonstrates the dichotomous effects of IFN-γ, in regulating the generation $CD4^+CD25^+$ Tregs during Th1 and Th2 responses, and the ability to promote or abrogate immune privilege.

Role of Th2 Cells in Corneal Allograft Rejection

Th2 $CD4^+$ T cells are defined by their expression of the transcription factor GATA-3 and the production of IL-4, IL-5, and IL-13 [144]. Effective protection against extracellular pathogens, such as helminthes, requires a Th2 response. It was once believed that a Th2 immune response would cross regulate a Th1 immune response, and vice versa [145]. Thus, it was hypothesized that by redirecting the host response towards a Th2 profile, the Th1 alloimmune response would be downregulated, resulting in reduced corneal allograft rejection [146]. However, the induction of atopic diseases, such as allergic conjunctivitis or allergic airway hyperactivity, dramatically increased the incidence and tempo of corneal allograft rejection [147-149]. The adoptive transfer of Th2 cells into SCID BALB/c mice resulted in 20% graft rejection. Interestingly, the addition of Th1 cells in the adoptive transfer resulted in 100% rejection [148]. Moreover, the depletion of IFN-γ in corneal allograft recipients results in $CD4^+$ T cells with a Th2 cytokine profile. It was observed that $CD4^+$ T cells that are specific for either mH or MHC alloantigens are easily suppressed by Tregs. However, if the $CD4^+$ Th2 cells are directed against both MHC and mH antigens, they become resistant to Treg suppression [143].

It is proposed that the induction of allergic immune responses promotes graft rejection by altering the systemic induction of immune privilege. This was first demonstrated in studies where allergic conjunctivitis was induced in one eye and a corneal allograft was placed on the allergic eye or on the non-sensitized contralateral eye. Both eyes succumbed to inflammation

and rejected the corneal allograft [150]. The recipients with allergic conjunctivitis produced elevated levels of IL-4 within the serum, and the induction of allergic conjunctivitis in IL-4R$^{-/-}$ mice resulted in the same incidence of corneal allograft rejection as non-sensitized WT corneal allograft recipients [151]. Further studies elucidated how IL-4 resulted in the dysregulation of $CD4^+CD25^+$ Treg mediated tolerance in corneal allograft recipients. The elevated IL-4 during an allergic response did not directly inhibit the suppressive function of Tregs, as $CD4^+CD25^+$ Tregs still maintained their suppressive function against $CD4^+$ Th1 effector cells. Rather, IL-4 rendered $CD4^+$ effector cells resistant to $CD4^+CD25^+$ Treg suppression [149]. Thus, on-going allergic diseases disable the tolerogenic axis by promoting an inflammatory reaction that cannot be suppressed by conventional Tregs.

Role of Th17 Cells in Corneal Allograft Rejection

Th17 $CD4^+$ T cells produce IL-17 and express the transcription factor RORγt [152, 153]. The Th17 lineage constitutes a branch of the adaptive immune system that has a function in the clearance of specific types of pathogens that require a massive inflammatory response and are not adequately dealt with by Th1 or Th2 immunity [152, 153]. This cell subset has been implicated in the pathogenesis of autoimmune diseases, including the ocular disease uveitis [154]. However, recent studies have shown IL-17 may be important for establishing immune privilege and promoting corneal allograft survival [155, 156]. Corneal transplants grafted into IL-17$^{-/-}$ hosts experienced a decreased rate but not incidence of allograft rejection [157]. Neutralization of IL-17A in BALB/c recipients resulted in 90% rejection of the B6 corneal allograft [155, 156]. It was demonstrated the IL-17 promoted the generation of Tregs as the addition of IL-17A to $CD4^+CD25^+$ Tregs licensed their suppressive function. In the absence of

IL-17, $CD4^+CD25^+$ Tregs lost the expression of cytotoxic lymphocyte antigen 4 (CTLA-4), GITR, and m-TGF-β, resulting in their reduced capacity to mediate a suppressive response [158]. Thus, corneal allograft survival may require Th17 cells during the inductive phase of corneal transplantation tolerance.

Role of T Regulatory Cells in Corneal Allograft Survival

$CD4^+CD25^+$ Tregs make up 5-10% of the $CD4^+$ T cell population and are defined by the expression of the transcription factor FoxP3 [159]. Tregs can be further characterized as either natural or induced. Thymus-derived Tregs have T cell receptors that are specific for autoantigens and thus, maintain immune tolerance to self and maintain homeostasis. However, conventional $CD4^+$ T cells, exposed to antigens in the periphery can express FoxP3 and gain suppressive functions that suppress immune responses towards foreign antigens [160]. $CD4^+CD25^+$ Tregs have an important role in corneal allograft survival [141, 161]. Studies on penetrating keratoplasty in mice have shown that recipients that accepted corneal allografts developed $CD4^+CD25^+$ Tregs with elevated levels of FoxP3 compared to hosts that had rejected their corneal transplants [161]. Moreover, the adoptive transfer of $CD4^+CD25^+$ T cells from corneal allograft acceptors into normal risk recipients enhanced allograft survival [161]. As mentioned earlier, IL-17A is required for the induction of $CD4^+CD25^+$ Tregs in corneal allograft recipients. These induced Tregs express the suppressive soluble molecules, TGF-β and IL-10. However, the optimal Treg suppressive capacity is cell contact-dependent and requires the molecules CTLA-4, GITR, and m-TGF-β to suppress effector T cell functions [158, 161]. Moreover, neutralization of IL-17A reduced the suppressive function of $CD4^+CD25^+$ T cells by limiting the expression of FoxP3 and the expression of cell membrane suppressive molecules.

This in turn, reduced the recipient's capability to sustain immune privilege and resulted in graft rejection [158].

CTLA-4 is a structural homologue of the costimulatory receptor CD28 and binds to the costimulatory molecules CD80 and CD86 on APCs. Due to its superior affinity, it was believed that CTLA-4 out-competes the activating receptor CD28 for binding to the costimulatory molecules [162]. Conditional knockout of the CTLA-4 gene in Tregs impairs their suppressive activity, resulting in splenomegaly and lymphadenopathy, ultimately leading to autoimmune gastritis [163]. Moreover, CTLA-4 may also affect the function of APCs, by down-modulating CD80/CD86 and inducing the immunoregulatory enzyme indoleamine-2,3-dioxygenase (IDO) [164, 165]. IDO is an enzyme in the tryptophan metabolism cascade that converts tryptophan to formylkynurenine and inhibits T effector cells [166].

GITR, a costimulatory receptor belonging to the TNF-receptor superfamily, is highly expressed on $CD4^+CD25^+$ Tregs, but is also constitutively expressed at low levels on $CD4^+CD25^-$ and $CD8^+$ T cells and is upregulated on these cell subsets upon activation [167]. The receptor, GITR Ligand (GITRL) is expressed on APCs, retinal pigment epithelial cells, Müller cells, and retinal photoreceptors [168, 169]. GITRL expression within the cornea is important for the protection of endothelial cells against $CD4^+$ T cell destruction. Infiltrating activated $CD4^+$ $GITR^+$ T cells bind to $GITRL^+$ endothelial cells and are converted to $FoxP3^+CD4^+CD25^+$ Tregs with suppressive function. However, if GITRL is blocked, naïve $CD4^+$ T cells become sensitized against the alloantigens expressed on the cornea and mediate endothelial cell killing [71].

TGF-β1 is an inhibitory cytokine that is important for peripheral immune homeostasis because of its anti-proliferative and anti-apoptotic effects [170]. Moreover, TGF-β1 plays a role

in the differentiation of $CD4^+$ T cells. *In vitro* stimulation of naive T cells in the presence of TGF-β1 leads to the induction of Foxp3 [171], and inhibition of Th1 differentiation [172]. Although the mechanism by which TGF-β1 suppresses immune reactions is not fully understood, its induction of $CD4^+CD25^+Foxp3^+$ Tregs and its inhibition of Th1 differentiation might contribute to its regulatory activity.

High Risk Corneal Transplantation Recipients

Patients with corneal dystrophies such as keratoconus or Fuchs' endothelial dystrophy have the lowest rejection rate of corneal transplant rejection [173, 174]. However, keratoconus and Fuchs' dystrophy constitute only 18% and 4% of the indications for corneal transplantation, respectively (Table 1). By contrast, patients with corneal inflammation, neovascularization, trauma or infections have lost immune privilege and are at considerable risk for corneal allograft rejection [36, 175-179].

Maintaining all of the factors that support immune privilege is imperative for the survival of corneal allografts. If any of these factors fails, the corneal allograft becomes susceptible to attack by the immune system and undergoes rejection. Certain conditions can abrogate one or more of the factors that support ocular immune privilege and subject the corneal allograft to a destructive inflammatory immune response, resulting rejection. Patients that have pre-existing conditions making them vulnerable to graft rejection are termed "High-Risk" allograft recipients. The conditions that predispose a patient to undergo corneal allograft rejection circumvent immune privilege by different mechanisms. The 'High-Risk' conditions that cause a corneal allograft to consistently fail are discussed below.

Prevascularized Eyes

As stated above, the cornea is normally avascular. The avascularity of the cornea is maintained by several anti-hemangniogenic factors such as VEGFR2, angiostatin, endostatin and thrombospondin-1. [180, 181] However, trauma or infection can cause the infiltration of both blood and lymph vessels. Corneal hemangiogenesis and lymphangiogenesis prior to corneal transplantation significantly increase the risk for rejection. Patients presenting with a prevascularized eye have a rejection rate of 50-100% [178]. The afferent lymphatic vessels

provide a conduit for APCs loaded with corneal alloantigens to leave the cornea and migrate to the regional lymph node. After the APCs initiate the immune response by interacting with the peripheral lymphocytes, $CD4^+$ T cells migrate out of the lymphoid tissue through the peripheral blood stream and migrate to the eye. The presence of blood vessels within the corneal tissue allows the infiltration of $CD4^+$ T cells into the graft site and leads to graft rejection [182]. In vascularized human corneas, the extent of lymphangiogenesis strongly correlates with the degree of neovascularization. Thus, an increase in blood vessels in the cornea is accompanied by an equal increase in lymph vessels [182].

The loss of immune privilege due to vascularization has been demonstrated in a mouse model where placing sutures into the cornea prior to surgery results in a significant increase in hemangiogenesis and lymphangiogenesis [183]. The increased vascularization promotes the infiltration of APCs into the cornea and consistently results in allograft rejection [184, 185]. However, neutralization of VEGF-A prevents hemangiogenesis and lymphangiogenesis and reduces the infiltration of APCs into the cornea, resulting in a steep decline in the incidence of graft rejection [183].

For many years it was believed that the loss of immune privilege was solely due to the presence of blood vessels. However, studies by Cursiefen and Chen determined that the presence of lymph vessels, not blood vessels, in the cornea led to the high incidence of graft rejection [186, 187]. The importance of lymph vessels for graft rejection was demonstrated when secretion of VEGF-C by blood vessel endothelial cells stimulated the growth of lymph vessels. Selectively blocking VEGF-C reduced lymphangiogenesis and corneal allograft rejection in both the prevascularized and normal risk recipients [181, 187]. Administration of $\alpha 5\beta 1$ integrin similarly inhibited lymphangiogenesis and reduced allograft rejection [188].

Furthermore, vascularization not only increases graft rejection through the migration of APCs, but also promotes the recruitment of T cells into the corneal tissue. Amescua et. al. observed that prevascularized eyes produced the chemokine CXCL1/KC, which in turn elicited the production of T cell recruiting chemokines CXCL9/MIG and CXCL10/IP-10 resulting in graft rejection [189]. However, this was specifically due to vascularization, as nonvascularized eyes did not express these chemokines. Neutralization of CXCL1 in prevascularized hosts decreased the level of CXCL9 and CXCL/10 and enhanced graft survival [189].

Ksander et. al. found that prevascularization of the corneal tissue induced donor-specific $CD8^+$ CTLs that were not generated in normal, avascular corneas [110]. The adoptive transfer of CTLs into T cell-deficient mice that subsequently received a corneal allograft resulted in 100% rejection [113]. This suggests that under high-risk conditions, the generation of CTLs can independently abrogate immune privilege and result in corneal allograft rejection.

Allergic Conjunctivitis

Allergic responses occur when the body produces an immediate hypersensitivity response to an allergen. Upon exposure to the allergen, B cells undergo isotype switching to preferentially produce IgE and Th2 cells produce IL-4, IL-5, and IL-13. Allergens bind to Fab moiety of IgE antibodies, whose FcE receptors are embedded in the mast cell membrane. The cross-linking that occurs when IgE antibodies bind to the same allergen results in mast cells degranulation and the release of histamines, leukotrienes, proteases, prostaglandins and cytokines.

It was previously believed that corneal allograft rejection was solely dependent on IFN-γ-producing Th1 $CD4^+$ T cells and that the deviation of the immune system to a Th2 pathway would block Th1 cells and thus, prevents graft rejection. This was supported by the observation

that the Th1-prone C56BL/6 mice have a higher incidence of graft rejection than Th2-prone BALB/c mice. However, tilting the immune response to a Th2 pathway has an opposite effect, as the depletion of IFN-γ or the induction of allergic conjunctivitis in BALB/c recipients exacerbates corneal allograft rejection [142, 150].

Some investigators have noted that patients with ongoing allergic conjunctivitis have an increased incidence of graft rejection [177, 190, 191]. This exacerbation of graft rejection was attributed to the inflammatory effects in a 'hot eye'. However, animal studies found that inducing allergic conjunctivitis causes a steep increase in the tempo and incidence in corneal allograft rejection [150, 151, 192]. Moreover, the exacerbation of corneal allograft rejection occurs even if allergic conjunctivitis is induced in the opposite eye and the corneal allograft is placed in the "quiet" eye that was not challenged with allergen and showed no clinical evidence of allergic conjunctivitis. Moreover, studies using a mouse model of allergic asthma revealed a similar exacerbation in corneal allograft rejection occurs in hosts with ongoing allergic diseases in the lungs [147]. These findings suggest that the loss of immune privilege due to allergy is not due to perturbation of the local ocular environment, but is caused by the alteration of the systemic immune response [142, 150]. Inducing allergic conjunctivitis with topical application of short ragweed extract enhances the level of Th2 cytokines. $CD4^+$ T cells isolated from atopic corneal allograft rejectors produced higher levels of IL-4 compared to non-allergic allograft recipients [148, 151]. The IL-4 produced during the allergic response binds to the IL-4 receptor on $CD4^+$ T effector cells and renders them resistant to Treg suppression resulting in allograft rejection [151].

Heightened Incidence of Graft Rejection in Previous Corneal Allograft Recipients

The need for multiple corneal transplants has increased worldwide [193, 194]. According to the Eye Bank Association of America, repeat corneal transplantation rose from 6.9% in 1992 to 12.1% in 2012 [23, 195]. This is an alarming perspective, because the patients who are at highest risk of graft rejection are those who require multiple corneal transplants. Patients who have rejected a corneal allograft may elect to have another corneal allograft. However, this comes with increased risk for graft rejection as the two year survival rate of subsequent corneal allografts drops from 90% for a primary graft, down to 70% for a second graft, 60% for a third graft, and continues to decrease with increasing numbers of transplants [176, 193, 196]. The rejection of the initial corneal transplant is an inflammatory response that introduces multiple risk factors that pre-dispose the recipient to have a higher incidence of graft rejection. However, the exact mechanism leading to the increased incidence of rejecting subsequent corneal allografts is not known.

It has been observed that the recognition of foreign human leukocyte antigen (HLA) antigens is the most important factor that determines the survival or rejection of solid tissue transplants, such as heart and kidney. As stated before, unlike other forms of solid tissue transplantation, corneal allografts are not routinely typed for the histocompatibility markers prior to transplantation, and yet under normal conditions, have a high acceptance rate [197]. Even under high-risk situations, histocompatibility matching does not improve corneal allograft survival. However, mounting evidence indicates that mH alloantigens evoke a stronger immune rejection response in both avascular and vascularized hosts than corneal allografts expressing MHC alloantigens [27, 198-200].

It has been disputed if the increased incidence of graft rejection in recipients of multiple corneal allografts is attributed to the presence of shared alloantigens presented on the first and subsequent donor corneal tissues. Prior sensitization revokes the rules of immune privilege as orthotopic transplantation of skin allografts expressing the same alloantigens as those found on corneal allografts placed onto the same host results in the destruction of the orthotopic corneal transplant, even if it is transplanted onto an avascular eye [201]. Thus, it has been widely speculated that the corneal transplant patient mounts an immune recall response when the second allograft is transplanted. The memory $CD4^+$ T cells from a patient receiving a second corneal allograft will recognize the MHC and mH molecules and mediate graft rejection [202]. However, since corneal tissues destined for transplantation are selected at random without HLA matching of the donor and the corneal allograft recipient, the likelihood that the corneal donor and the recipient share MHC or mH antigens is remote. We initially hypothesized that the heightened incidence of subsequent allograft rejection was due to the recognition of shared alloantigens. However, this study will show that an immune memory response is not the reason for the heightened incidence of graft rejection. Rather, the corneal transplantation procedure itself revokes immune privilege and results in a high incidence of graft rejection in both eyes.

Although the rejection of a corneal previous corneal allograft is the highest risk factor in the revocation of immune privilege in keratoplasty patients [203], this study provides evidence that the inflammatory effects arising from a rejected corneal allograft are not involved in the abolition of immune privilege of a subsequent corneal allograft. This study provides evidence that the corneal transplantation procedure converts the patient to become a 'high-risk' for rejecting subsequent corneal allografts by altering and abolishing the immunosuppressive microenvironment. Streilein previously observed that the corneal transplantation procedure, not

just the rejection process, abolishes local immune privilege and inhibits the induction of ACAID [203]. That study also demonstrated that surgical maneuvers involved in corneal transplantation, specifically circumferentially incising and the placement of sutures into the host's corneal tissue, affected the induction of ACAID [203].

Streilein et. al. observed that the insertion of sutures induced a robust infiltration of blood vessels into the cornea and a concomitant infiltration of LCs into the central cornea [203]. Recipients with robust blood vessels in their corneas could not support the induction of ACAID and produced a pronounced DTH response [203]. The postoperative infiltration of blood vessels has been closely associated with the sequential rejection of a corneal allograft [182, 187]. During rejection, blood and lymphatic vessels infiltrate into the transplanted cornea and establish a highly vascularized microenvironment [204-206]. Thus, the immune system gains accessibility to the subsequent corneal allograft and mediates rejection. As discussed earlier, multiple studies have investigated how the infiltration of blood, and more importantly lymphatic vessels, efficiently sensitizes a recipient towards alloantigens presented on the corneal allograft [180, 182, 183, 207]. Streilein and co-workers also addressed how recapitulating the excision of the recipient's cornea, through a circumferential incision, inhibited the induction of ACAID [203]. This was specifically attributed to the effects of a circumferential incision, as an X-shaped incision still resulted in the induction of ACAID [203]. The loss of immune privilege was attributed to severing the corneal nerves and the diminished secretion of soluble immunosuppressive molecules by the cornea into the AqH. Our study addressed how these two maneuvers promote corneal allograft rejection and if the loss of immune privilege is extended to the contralateral eye.

Current Investigations

First time uncomplicated corneal transplants have a 90% acceptance rate. This high acceptance rate is attributed to ocular immune privilege [208, 209]. However, patients with prevascularized corneas, dry eye, allergic conjunctivitis or ocular inflammation have compromised immune privilege, and thus have an increased incidence and severity of graft rejection [178, 183, 184]. Previous studies have elucidated the mechanism of how prevascularization and allergic conjunctivitis culminate in a high incidence of graft rejection [148, 149, 182]. However, the population at greatest risk of rejection is the one that incudes patients that have rejected a previous corneal allograft [196, 205, 206, 210]. Little is known about how and why the risk of corneal graft rejection soars in patients who receive a second or third corneal transplant.

The main scope of this study is to determine why repeat corneal allograft recipients, especially patients that require bilateral transplantation, have an increased risk of rejection. This was tested using a well-established mouse model in which the corneal allograft donors and hosts are mismatched at both the MHC and all known mH gene loci. This donor-host combination mimics the disparity that occurs when patients receive corneal transplants that are selected at random, with no effort to match the histocompability genotype between the graft donor and the recipient. Our first aim was to determine if prior sensitization to shared alloantigens or corneal-specific antigens promoted the heightened incidence of rejection of subsequent corneal allografts. The results presented in this study demonstrate that the enhanced incidence of subsequent corneal allograft rejection is not due to a memory recall response; rather the corneal allograft procedure itself perturbs immune privilege and promotes corneal allograft rejection. Further studies

examined how each aspect of the corneal transplantation procedure impacted corneal allograft immune privilege, not only in the surgically manipulated eye, but also in the contralateral eye.

Our first aim was to determine what aspect of the corneal transplantation procedure promoted the increased incidence of graft rejection. We found that a circumferential incision through the epithelium and stromal layers ablates the innervation processes in the central cornea and promotes graft rejection in both eyes. This study focused on how damage to the central corneal nerves altered the immune privilege status in the contralateral eye. The nerves within the cornea, and the mediators they produce, have a profound effect on the immune privilege of the eye. The results presented in this study demonstrate that the microenvironment of the contralateral eye has an altered expression of immunoregulatory neuropeptides. The next portion of the study investigated how the alteration of those mediators, namely SP, would promote corneal allograft rejection.

Corneal allografts require $CD4^+CD25^+$ Tregs to suppress the inflammatory DTH response mediated by $CD4^+$ T effector cells. It is not known how the damage to the corneal nerves disrupts tolerance and leads to alloantigen driven inflammation. The final aim of this study was to determine how a circumferential wound to the corneal would mediate a systemic loss of immune privilege. The present study demonstrates that circumferential incisions lead to enhanced production of SP, which can inhibit both the induction and function of $CD4^+CD25^+$ Tregs, thereby rendering the host susceptible to $CD4^+$ T cell-mediated corneal allograft rejection.

CHAPTER TWO

MATERIALS AND METHODS

Animals

C57BL/6 (H-2^b) and BALB/c (H-2^d) were purchased from the UT Southwestern Mouse Breeding Facility. For grafting experiments, eight- to ten- week old female BALB/c and C57BL/6 (B6) mice were purchased from Taconic Farms (Germantown, NY), A/J (H-2^a) and C3H/HeJ (H-2^k) mice were purchased from Jackson Laboratory (Bar Harbor, ME). 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.

In vivo Spantide II, Substance P, and α-MSH Treatment

Mice were treated with intraperitoneal (i.p.) injections of 72 μg Spantide II (Sigma-Aldrich) reconstituted in PBS daily starting the day prior to trephining or corneal allografting, and continued everyday thereafter. Injections of 1 pg/dose SP (Sigma-Aldrich) were administered in the subconjuctiva at three radial points around the eye were performed using a 20 gauge stainless steel needle attached to a 100 ml glass syringe (Hamilton Company, Reno, NE) or through the venous route by injecting into the tail vein. 2 μg synthesized α-MSH (Bachem, Torrance, CA) in PBS was administered i.p. every three days from day -15 to day +1.

Local Adoptive Transfer Assay

The Local Adoptive Transfer (LAT) assay to test the suppression of $CD4^+CD25^+$ Tregs has been described previously [211]. $CD4^+$ $CD25^+$ T cells (1 x 10^6 cells) and $CD4^+$ T cells (1 x 10^6 cells) were isolated on day 21 from BALB/c mice that previously received a C57BL/6 allograft, and adherent BALB/c splenocytes pulsed with C57BL/6 spleen cells (1x10^6 cells) were mixed in a 1:1:1 ratio of HBSS in a total volume of 20 μl and were injected into the left ear pinna of naïve BALB/c mice. The right ears served as negative controls and were injected with 20 μl of HBSS without cells. The suppressive function of $CD4^+CD25^+$ T cells was demonstrated by the inhibition of the ear swelling responses mediated by immune $CD4^+$ T cells. In some experiments, one pg. of SP (20 μl) was co-injected with the mixed cell populations. Results were expressed as B6- specific ear swelling response = (Experimental ear 24 hr measurement – Experimental ear 0 hr measurement) – (Negative control ear 24 hr measurement – Negative ear 0 hr measurement). Ear swelling was measured 24 hours later to assess DTH.

Orthotopic Corneal Transplantation

BALB/c mice received full thickness orthotopic corneal grafts from C57BL/6, C3H, A/J, or BALB/c mice as previously described [126]. 60 days after BALB/c hosts undergo allograft rejection or receive a syngeneic graft, the primary A/J, C3H, or BALB/c graft is surgically removed and replaced with a subsequent B6 allograft or BALB/c syngeneic graft. Alternatively, BALB/c hosts received a C57BL/6 or BALB/c graft in the unmanipulated eye. Corneal grafts were examined 2-3 times a week for opacity, neovascularization and edema with a slit-lamp biomicroscope (Carl-Zeiss, Oberkochen, Germany). Degree of opacification ranged between 0 and 4+; with 0 = clear; 1+ = minimal superficial opacity; 2+ = mild deep stromal opacity with pupil margin and iris visible; 3+ = moderate stromal opacity with pupil margin visible, but iris structure obscured; and 4+ = complete opacity, with pupil and iris totally obscured. Clinical

scores based on opacity were assessed until the allograft was determined rejected. Corneal grafts were considered rejected upon two successive scores of 3 [212]. No immunosuppressive drugs or topical corticosteroids were used in any of these experiments.

Intrastromal Suturing and Corneal Surface Incisions

A 2.0 mm circular trephine was used to make 360° shallow incisions that penetrated half way into the corneal stroma and did not penetrate into the AC. The circular incisions were made by applying slight pressure while twisting 10-15 times over the corneal surface. Vascularized, high-risk graft beds were produced as previously described [199]. Three interrupted sutures (11-0 nylon, 50-μm diameter needle; Sharpoint, Vanguard, Houston, TX) were placed through the central cornea of the right eye of recipient mice 2 weeks prior to orthotropic corneal transplantation. Sutures entered the anterior one-third to one-half of the stroma and did not penetrate into the AC. X-shaped incisions were created by cutting two 1.0 mm perpendicular lines through the epithelium and stroma of the central cornea.

Corneal Nerve Imaging

Enucleated eyes from mice were digested in Dispase II (Gibco, Grand Island, NY) for 2 hours at room temperature then fixed in 4% paraformaldehyde for 40 minutes at room temperature. Corneas were excised under a surgical scope and washed with PBS. Tissues were permeabilized and blocked in 1% BSA in PBS and 0.2% Triton X-100 for 2 hours at room temperature. Corneas were incubated with rabbit anti-mouse β III tubulin primary antibody (TUJ_1) IgG (Covance, Richmond, CA) at a dilution of 1:300 for 16 hours at 4°C. Corneas were stained with Propidium Iodide (eBiosciences, San Diego, CA) at a dilution of 1:200 and Alexa 488 goat anti-

rabbit IgG secondary antibody (Invitrogen Carlsbad, CA) at a dilution of 1:300 for 2.5 hours at room temperature. Images were captured on a Leica DM IRE2.

Quantitative Real Time PCR (q-RT-PCR)

BALB/c mice received a circumferential corneal incision in the right eye. The anterior tissue, cornea and iris, of the incised or unmanipulated eye from 4 donors were excised and placed on ice in 700 µl cRPMI. The tissue was disrupted using a mortar and pestle followed by homogenization with a 25 gauge needle and syringe. Total RNA was isolated using the Qiagen RNEasy Isolation Kit. Real-time PCR was performed using the RT^2 First Strand and RT^2 SYBR Green kits with pre-formulated primers for Tac 1, pro-opiomelanocortin (Pomc), VIP, Calca, Sst, and GAPDH (SA Biosciences, Frederick, MD). The results were analyzed by the comparative threshold cycle method and normalized with GAPDH as an internal control.

Neuropeptide Protein Quantification by Enyzme Immunoassay

BALB/c mice received a circumferential corneal incision in the right eye. The anterior tissue, cornea and iris, of the trephined or unmanipulated contralateral eye from 4 donors were excised and placed into 400 µl of PBS containing Sigma-Fast Protease inhibitor cocktail on ice (Sigma Aldrich, St. Louis, MO). Each sample was processed on ice with 4 cycles of 10 seconds sonication and 1 minute rest between each pulse to allow the sample to cool. The lysates were then centrifuged at 10,000 RPMI for 5 minutes at 4^o C. The supernatants were collected and stored at 4^o C until assayed. Enzyme Immunoabsorbant Assays (EIAs) for Substance P (Cayman Chemical, Ann Arbor, MI), α-MSH, VIP, Somatostatin, and CGRP (Phoenix Pharmaceuticals, Burlingame, CA) were performed on corneal tissue lysate diluted at 1:10 in EIA solution according to the manufacturers' instructions.

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. EIA data are represented as the mean. LAT assay data was analyzed using a two tailed Student's t-test [213]. *P* values less than 0.05 were considered significant.

CHAPTER THREE

RESULTS

Corneal allograft rejection promotes a heightened incidence of rejection of a subsequent unrelated corneal allograft.

We used a well-established murine model of orthotopic transplantation to test the hypothesis that immune rejection of a corneal transplant increased the risk for rejection of subsequent corneal transplants, even those from donors unrelated to the first corneal transplant. C57BL/6 (B6) (H-2k^b) mice served as corneal transplant donors and BALB/c (H-2k^d) mice as corneal transplant recipients. The B6 mouse differs from the BALB/c recipient at the entire MHC and all known minor H gene loci and thus resembles the typical disparity that occurs when patients receive corneal transplants selected at random without any effort at MHC matching. Grafts were assessed by grading the degree of clarity of the corneal allograft over a period of 60 days. Grafts remaining clear at day 60 were deemed long-term survivors as the incidence of graft rejection after day 60 is less than 1% (unpublished findings). In this donor-host combination, 50% of the corneas remain clear and are considered accepted, while the other 50% undergo immune rejection. This represents the baseline of rejection in recipients without any predisposing factors that break immune privilege.

The first aim of this study was to determine if rejection of a corneal allograft disrupts local immune privilege and results in the increased incidence of graft rejection of an unrelated corneal allograft. Thus, we used A/J (H-2k^a) or C3H (H2-k^k) mice as corneal allograft donors for primary corneal transplants and B6 (H-2k^b) mice as the donors of subsequent corneal allografts. These four mouse strains (A/J, C3H, BALB/c, and B6) do not share any major or minor histocompatibility gene loci.

A/J and C3H corneal allografts were rejected within 4 weeks (A/J rejection = 100%; C3H rejection = 100%) (Figure 3). The BALB/c recipients that rejected an A/J or C3H allograft were rested for 60 days after the last graft was declared rejected. This time span allows the acute stage of the inflammatory rejection process to subside. The rejected A/J or C3H allograft was removed from the BALB/c recipient and a B6 cornea was transplanted onto the graft bed of the right eye (Figure 4A). B6 allografts underwent 100% rejection when placed onto a graft bed that had previously rejected an A/J (rejection = 100%, median survival time (MST) = 16 days) or C3H (rejection =100%, MST = 7 days) corneal allograft. Both the incidence and tempo of rejection was increased compared to B6 allografts transplanted onto naive BALB/c recipients (rejection = 50%, MST = 46 days) (Figure 4B). These results suggest the rejection of a corneal allograft alters the local ocular environment and disrupted the immune privilege status of a subsequent unrelated corneal allograft placed onto the same eye. Moreover, these results indicate that the increased incidence of rejection for second corneal transplants is not due to a memory or recall response to donor histocompatibility antigens expressed on both grafts, as none of the corneal allograft donors or the BALB/c recipients shares any major or minor histocompatibility antigens.

We next turned our attention to the fate of a corneal transplants grafted to a host that had rejected a previous unrelated corneal transplant in the opposite eye. That is, does rejection of a corneal transplant in one eye rob the opposite eye of its immune privilege? A/J or C3H corneal allografts were transplanted onto the right eye of BALB/c recipients. The BALB/c recipients were rested for 60 days after the last graft was declared rejected. The rejected A/J or C3H corneal allografts were not removed from the right eye and B6 corneal allografts were transplanted onto the left eyes (Figure 5A). All of the B6 corneal allografts underwent rejection

when placed onto the unmanipulated graft bed of recipients that had rejected either A/J corneal allografts (rejection = 100%; MST = 16) or C3H corneal allografts (rejection = 100%; MST = 12.5) in the opposite eye. This represents a significantly increased incidence and tempo of rejection compared to the rejection of B6 corneal allografts transplanted onto naïve BALB/c recipients (rejection = 50%, MST = 46; $P < 0.001$) (Figure 5B). These results suggest that rejection of a corneal allograft alters the ocular environment in both eyes and abolishes immune privilege to subsequent unrelated corneal allografts placed onto the contralateral eye.

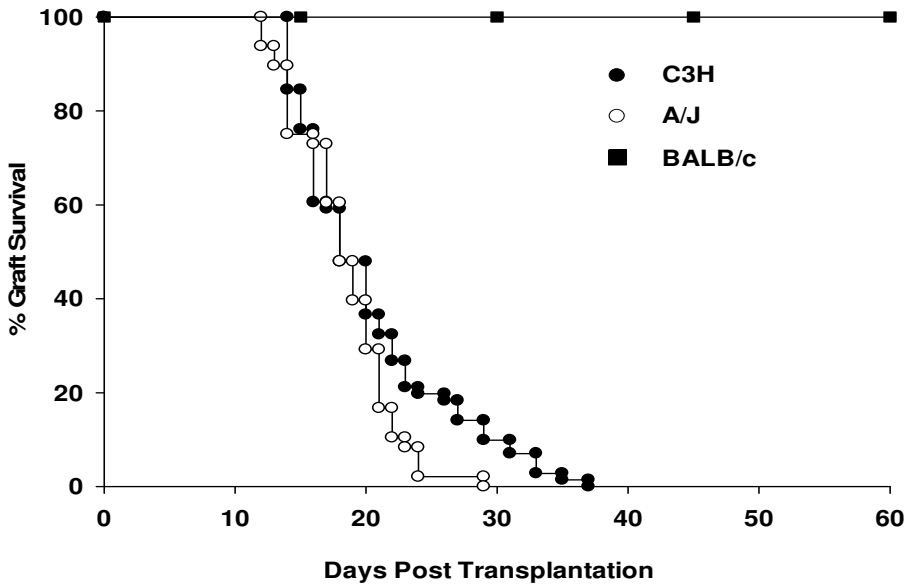
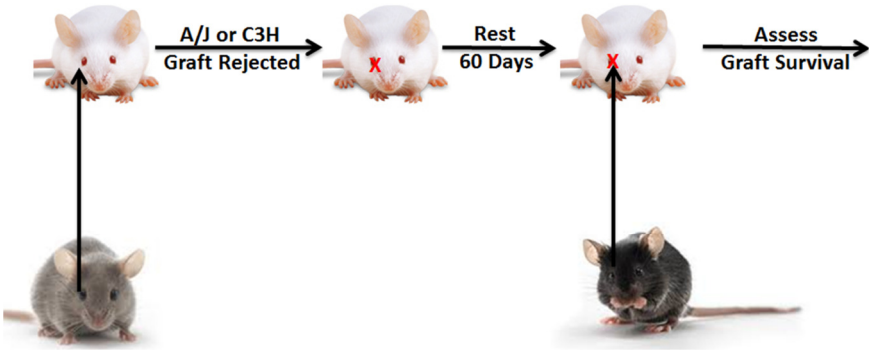


Figure 3. Survival status of initial corneal grafts. BALB/c recipients rejected corneal allografts or accepted corneal syngeneic grafts. BALB/c recipients received A/J or C3H corneal allografts or BALB/c syngeneic grafts onto one eye. The recipients of A/J corneal allografts (○; N = 48) had a 100% rejection rate. The recipients of C3H corneal allografts (●; N = 71) had a 100% rejection rate. The recipients of the syngeneic BALB/c graft (■; N = 50) universally accepted their syn-grafts.

A.



B.

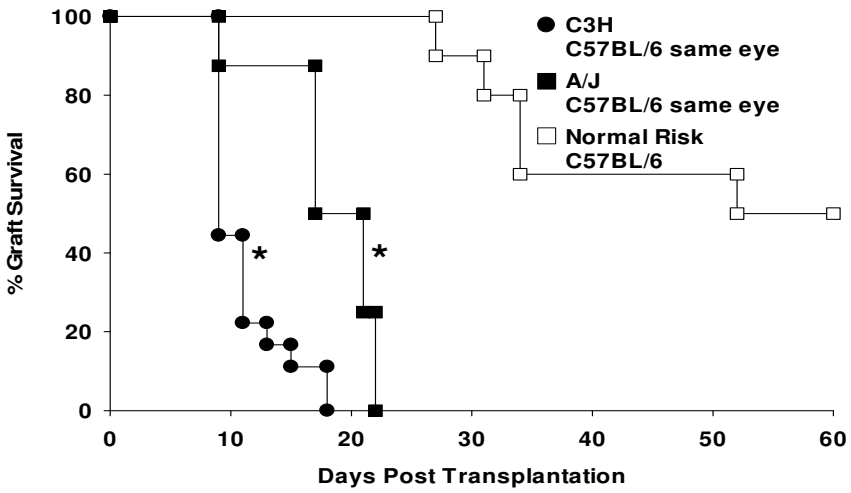
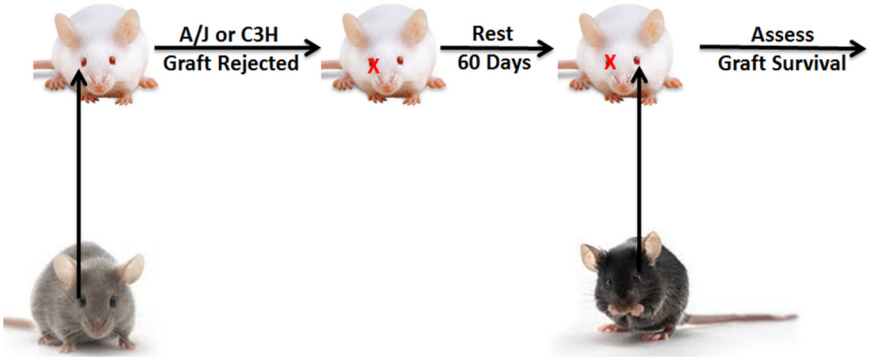


Figure 4. Corneal allograft rejection increases rejection incidence of a subsequent unrelated allograft transplanted onto eyes in which corneal allografts have undergone rejection. A.) Experimental design where BALB/c recipients received a C3H or A/J orthotopic corneal allograft in the right eye. 60 days after the last graft underwent rejection, C3H or A/J grafts were removed and were replaced with B6 corneal allografts. The B6 allografts were observed for 60 days or until declared rejected. B.) BALB/c recipients that did not receive corneal allografts prior to receiving B6 transplants (□; N = 10) had a 50% B6 corneal allograft rejection rate with an MST of 46 days. The recipients that rejected A/J corneal allografts (■; N = 8) or C3H corneal allografts (●; N = 18) rejected 100% of the B6 corneal allografts transplanted onto the same eye with an MST of 16 and 7 days, respectively. *P < 0.001

A.



B.

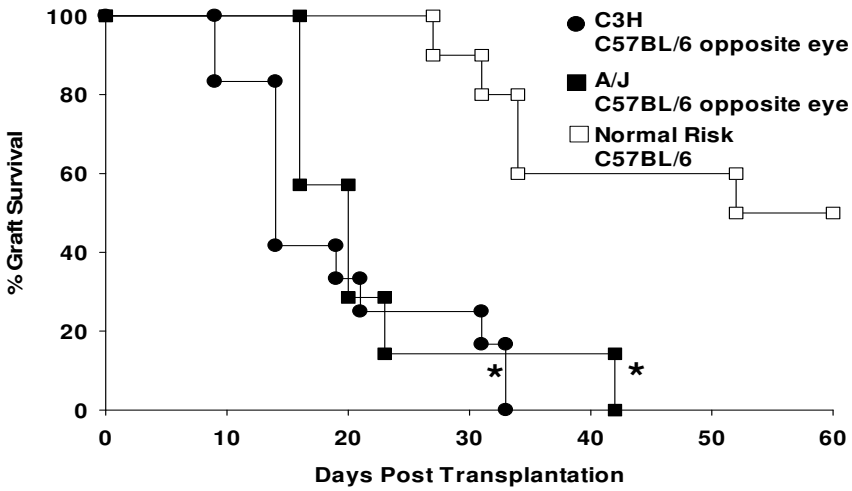


Figure 5. Corneal allograft rejection increases the rejection incidence of subsequent unrelated corneal allografts transplanted onto the contralateral eye. A.) Experimental design where BALB/c recipients receive either C3H or A/J orthotopic corneal allografts in the right eyes. 60 days after the last graft underwent rejection, B6 allografts were placed onto the contralateral left eyes of BALB/c recipients. B6 grafts were observed for 60 days or until declared rejected. B.) BALB/c recipients that did not receive corneal allografts prior to receiving B6 corneal allografts (□; N = 10) had a 50% B6 corneal allograft rejection rate with an MST of 46 days. The recipients that rejected A/J allografts (■; N = 7) or C3H corneal allografts (●; N = 12) rejected 100% of their B6 corneal allografts transplanted onto the contralateral eyes (MST = 16 and 12.5 days, respectively).*P < 0.001

Orthotopic corneal transplantation itself abolishes immune privilege, even in the case of syngeneic corneal transplants that do not undergo immune rejection.

We next tested the hypothesis that the orthotopic transplantation procedure itself abolishes immune privilege. This was tested by transplanting syngeneic BALB/c orthotopic corneal transplants onto the right eyes of BALB/c recipients, and after sixty days, replacing these grafts with B6 corneal allografts (Figure 6A). As expected the syngeneic BALB/c corneal allografts remained clear and healthy throughout the entire 60 day observation period (Figure 1). However, B6 corneal allografts that replaced the BALB/c syngeneic grafts were rejected at an increased incidence and tempo (rejection = 100%, MST = 15.5 days) compared to an unmanipulated BALB/c recipient (rejection = 50%, MST = 46 days) (Figure 6B). This suggests that orthotopic corneal transplantation procedure itself, irrespective of graft rejection or graft survival, abolishes immune privilege and leads to a sharp increase in the rejection of subsequent unrelated corneal allografts

Additional experiments tested the hypothesis that the orthotopic corneal transplantation procedure also abolished immune privilege in the unmanipulated opposite eye. As before, syngeneic BALB/c grafts were transplanted orthotopically and the mice were rested for 60 days. Rather than removing the syngeneic graft, it was left in place and instead, a B6 corneal allograft was transplanted onto the opposite, unmanipulated left eye (Figure 7A). The B6 grafts underwent rejection at an increased incidence and tempo (rejection = 87.5%, MST = 20 days) compared to B6 corneal allografts transplanted onto unmanipulated BALB/c recipients (rejection = 50%, MST = 46 days) (Figure 7B). These results suggest that the surgical procedure alone is responsible for the loss of immune privilege and heightened incidence and tempo of subsequent corneal allografts in either eye.

A.

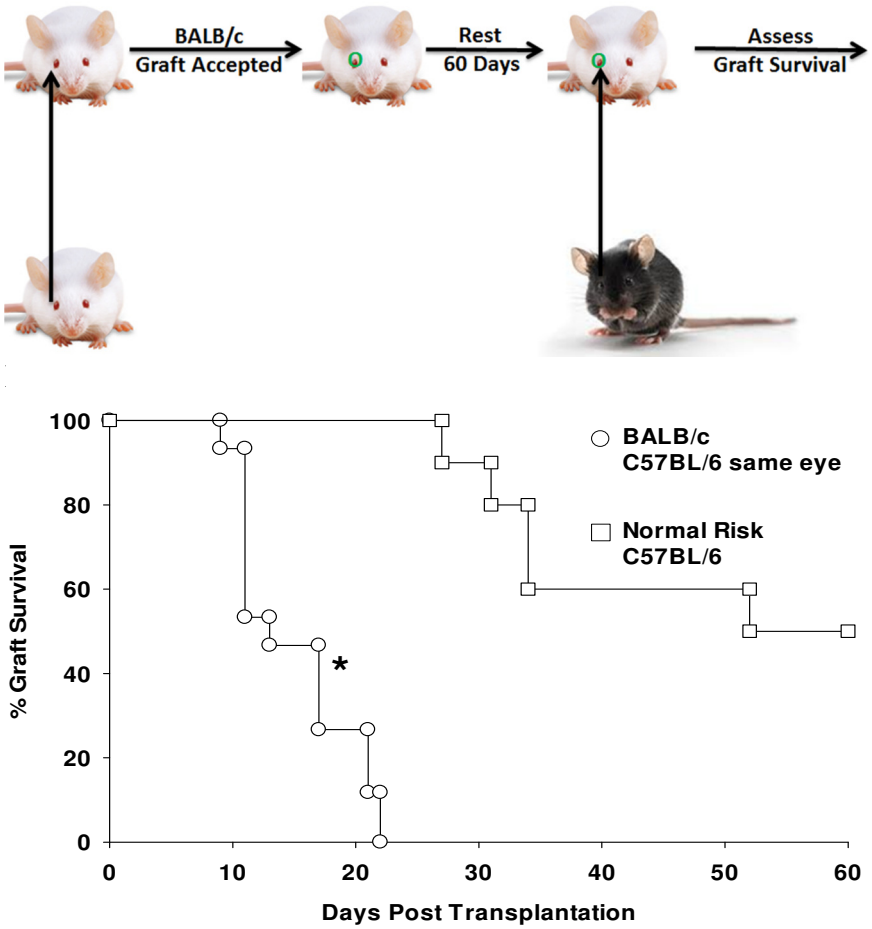


Figure 6. Syngeneic corneal grafts increase the incidence and tempo of rejection of subsequent allografts transplanted onto the same eye. A.) Experimental design in which BALB/c recipients receive a syngeneic BALB/c orthotopic corneal graft in the right eye. Sixty days later, syngeneic grafts were removed and were replaced with B6 corneal allografts. B6 allografts were observed for 60 days or until they underwent rejection. B.) BALB/c recipients that did not receive syngeneic BALB/c corneal grafts prior to receiving B6 corneal allografts (□; N = 10) had a 50% B6 corneal allograft rejection rate with an MST of 46 days. The recipients that accepted BALB/c syngrafts (○; N = 15) rejected 100% of the B6 corneal allografts transplanted onto the same eye with an MST of 15.5 days. *P < 0.001

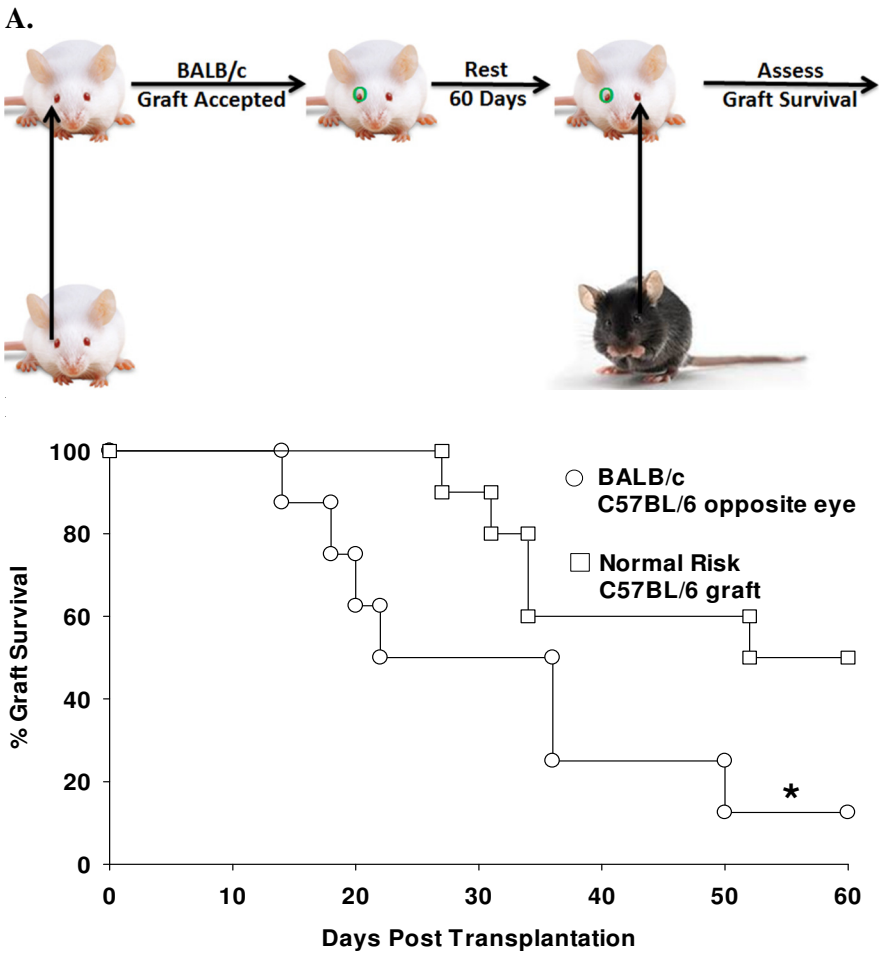


Figure 7. Syngeneic corneal grafts abolish immune privilege leading to increased rejection of subsequent corneal allografts placed onto the opposite, unmanipulated eyes. A.) Experimental design in which BALB/c recipients received syngeneic BALB/c orthotopic corneal grafts in the right eyes. Sixty days later, B6 corneal allografts were placed onto the contralateral eyes of BALB/c recipients. B6 corneal allografts were observed for 60 days or until they underwent rejection. B.) BALB/c recipients that did not receive syngeneic BALB/c corneal grafts prior to receiving B6 corneal allografts (□; N = 10) had a 50% B6 corneal allograft rejection rate with an MST of 46 days. The recipients that accepted BALB/c syngrafts (○; N = 8) rejected 85.72% of the B6 corneal allografts that were transplanted onto the contralateral eye (MST = 20 days). *P<0.05

The corneal transplantation procedure abolishes immune privilege to corneal alloantigens but has no effect on putative cornea-specific antigens.

Some tissue-specific antigens are not expressed in the thymus during T cell development and as such, T cells bearing T cell receptors for such tissue-specific antigens do not undergo clonal deletion in the thymus. In this regard, it has been proposed that T cells specific for certain retinal antigens escape clonal deletion during T cell development in the thymus and that these T cells can mediate autoimmune uveitis. Along the same lines, penetrating injuries to the eye can on occasion provoke ocular inflammation in both the damaged eye and the contralateral unaffected eye – a condition termed sympathetic ophthalmia (SO) [214-216]. It is hypothesized that the limited exposure of eye-specific antigens to the periphery renders these self-antigens immunogenic. We considered the hypothesis that a similar release of tissue-specific antigens, in this case cornea-specific antigens, would provoke an immune response against corneal antigens on subsequent corneal allografts. Thus, the breakdown of the ocular-blood barrier permits the release of these eye-specific antigens to the peripheral immune system and induces an inflammatory response in the same eye and the sympathizing eye [216, 217]. To determine if the enhanced incidence of graft rejection was due to an immune reaction against eye-specific antigens, recipients were never challenged with a graft expressing alloantigens. Rather recipients were challenged with syngeneic grafts and the survival of a subsequent syngeneic corneal graft placed on the same eye or the contralateral eye was evaluated.

BALB/c syngeneic grafts were transplanted onto the right eyes of naïve BALB/c recipients and as expected, accepted in 100% of the recipients. Sixty days later second BALB/c grafts were transplanted onto the previously grafted right eyes (Figure 8A) or onto the unmanipulated left eyes (Figure 8C). All of the subsequent BALB/c syngeneic grafts were

accepted when transplanted to eyes of BALB/c mice that had previously received either BALB/c syngeneic grafts on the same eye (rejection = 0%) (Figure 8B) or to the unmanipulated opposite eye (rejection = 0%) (Figure 8D). The survival of the syngeneic grafts placed onto previous corneal graft recipients recapitulated what occurred when syngeneic BALB/c grafts were placed onto an unmanipulated mouse (rejection = 0%) (Figure 3). This suggests that the corneal transplantation procedure does not induce the exposure of eye-specific antigens and does not provoke an immune response to corneal tissue antigens.

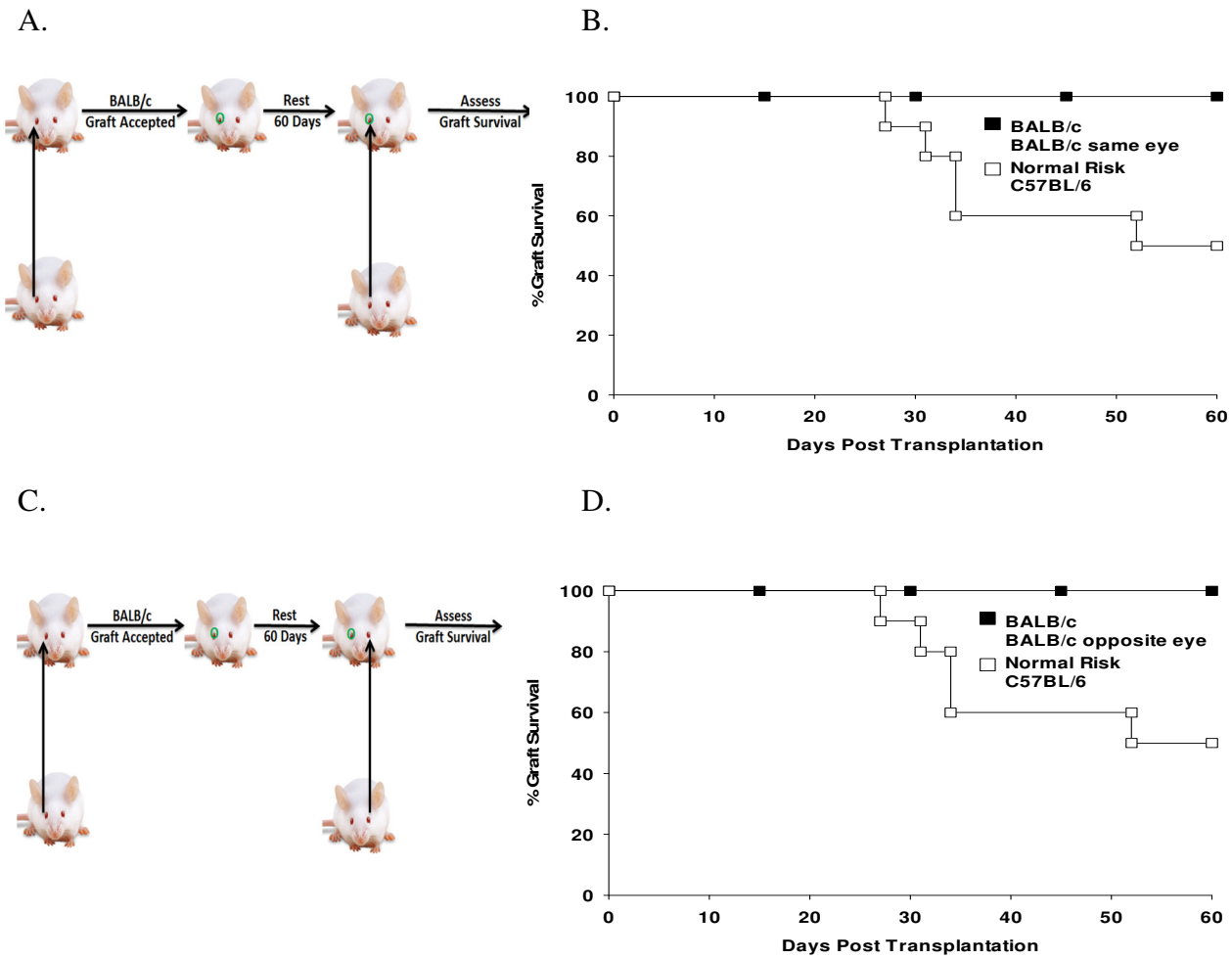


Figure 8. BALB/c syngeneic grafts do not elicit an auto-antigenic immune response towards eye-specific antigens. A.) Experimental design in which syngeneic BALB/c corneal grafts are transplanted orthotopically to the right eyes of BALB/c recipients. Sixty days later, BALB/c syngeneic grafts are transplanted onto the same eyes of the BALB/c recipients. BALB/c graft survival is assessed for 60 days or until declared rejected. B.) BALB/c recipients that did not receive syngeneic BALB/c corneal grafts prior to receiving B6 corneal allografts (■; N = 10,) had a 50% BALB/c corneal graft rejection rate with an MST of 46 days. The acceptance of the initial BALB/c syngeneic corneal grafts (□; N = 12) did not affect the acceptance of subsequent syngeneic BALB/c grafts placed in the same eye (0% rejection). C.) Experimental design in which syngeneic BALB/c orthotopic corneal grafts were transplanted to the right eyes of BALB/c recipients. Sixty days later, BALB/c syngeneic grafts were placed onto the contralateral eyes of BALB/c recipients. BALB/c graft survival was assessed for 60 days or until the grafts underwent rejection. D.) BALB/c mice that did not receive a syngeneic BALB/c corneal graft prior to receiving a B6 transplant (■; N = 10) had a 50% BALB/c corneal graft rejection rate with an MST of 46 days. The presence of an initial BALB/c corneal graft (□; N = 9) on one eye did not affect the survival of subsequent syngeneic BALB/c corneal grafts placed into the contralateral eye (0% rejection).

The corneal transplantation procedure itself abolishes local and systemic immune privilege

The presence of a primary syngeneic corneal graft leads to a heightened incidence of rejection towards subsequent corneal allografts. This heightened incidence of graft rejection was seen not only in the eye that received the graft but also in the unmanipulated contralateral eye. This suggested that the manipulation of the cornea during the transplantation procedure inhibited one or multiple factors that contribute to ocular immune privilege in both eyes.

In corneal transplantation, the central portion of the diseased cornea is removed and replaced with a clear, healthy cornea. In the first step, a surgical instrument with a cylindrical blade, referred to as a trephine, is placed onto the recipient's cornea to create a circumferential incision through the epithelium and stromal layers. This creates a guideline for the corneal surgeon to excise and remove the entire recipient's corneal tissue. The exposed anterior chamber and remaining recipient's peripheral corneal tissue create a graft bed into which the corneal transplant is placed. The second step of the corneal transplantation procedure is securing the donor's corneal tissue to the recipient's graft bed. The donor's tissue is sized to cover the entire anterior chamber and secured onto the recipient's peripheral corneal tissue by sewing very fine sutures through the donor's corneal tissue and the recipient's graft bed. After the tissue has healed into the recipient's graft bed, the sutures are removed.

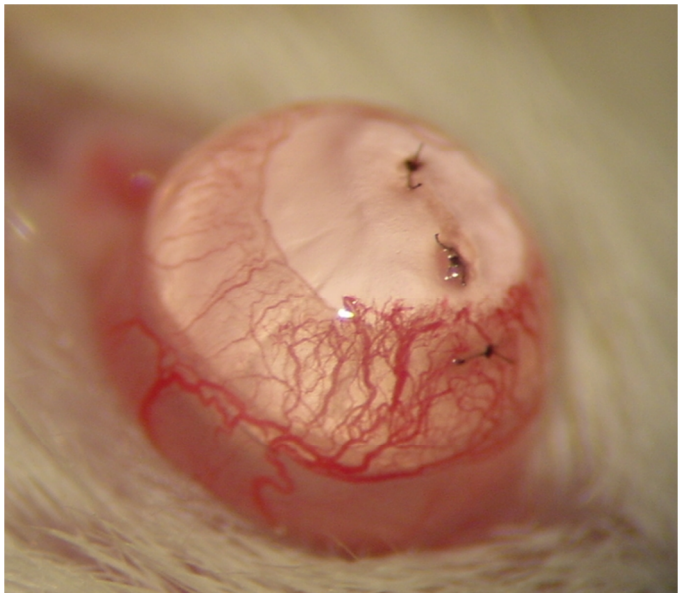
We wanted to determine which of these two manipulations performed during the corneal transplantation procedure instigated the loss of immune privilege in both eyes. To determine the effect of securing a transplant onto a recipient without performing the entire corneal transplantation procedure, three intrastromal 11-0 sutures were sewn into the left eyes of BALB/c mice (Figure 9A). Seven days later, the sutures were removed and the host's cornea was removed and replaced with a B6 corneal allograft. In other mice, B6 corneal allografts were

transplanted orthotopically to the eye opposite of the sutured eye. B6 corneal allografts transplanted onto the sutured eye were quickly rejected (rejection = 100%, MST = 18 days). By contrast, B6 corneal allografts transplanted onto the unsutured eyes of BALB/c mice that had sutures in the opposite eye displayed the same incidence and tempo of graft rejection (rejection = 50%, MST = 33 days) as mice that had not been treated with sutures (rejection = 50%, MST = 34 days) (Figure 9B). These results suggest that the suturing step of orthotopic corneal transplantation abolishes local immune privilege but does not affect the immune privilege of the opposite eye. Thus, the suturing step of orthotopic corneal transplantation does not contribute to the sympathetic loss of immune privilege that occurs in mice subjected to multiple corneal transplants.

We next evaluated the effect of cutting the corneal tissue has on the maintenance of immune privilege and corneal allograft survival. Accordingly, a 2.0 mm corneal trephine was utilized to create circumferential incisions around the central cornea of the left eyes of BALB/c mice. The incision penetrated through the epithelium and the upper portion of the corneal stroma, leaving the underlying endothelial layer intact (Figure 10A). Fourteen days later, B6 corneal allografts were transplanted onto the left eyes or untreated right eyes. The results revealed that corneal incisions led to a sharp increase in corneal allograft rejection in both eyes. B6 corneal allografts transplanted onto the incised eye underwent rejection in 85.8% of the mice (MST = 18 days) and with B6 cornea allografts transplanted onto the non-incised eyes (Rejection = 100%, MST = 25 days) (Figure 10B). The enhanced rejection was due to allosensitization, as circumferential incisions did not affect the survival or health of syngeneic corneal grafts transplanted onto the contralateral eyes of mice subjected to trephine incisions of the cornea (Figure 10B).

These results invited us to consider the hypothesis that the loss of immune privilege in the non-incised eye was due to the trauma induced by creating a general corneal surface incision, or specifically due to the destruction caused by creating a circumferential corneal surface incision. We introduced two 1.0 mm incision marks in the shape of an X in the left eye and transplanted B6 corneal allografts onto the right eyes fourteen days later (Figure 10C). B6 corneal allografts placed into the opposite eyes having X-shaped incisions were not affected and displayed graft survival that was not significantly different from unmanipulated hosts (rejection = 42%, MST = 36 days compared to 50% rejection, MST = 46 days respectively) (Figure 10D). These results suggested that the geometry of the corneal incisions had a profound effect on immune privilege. That is, 360° incisions abolished immune privilege, while simple linear incisions had no significant effect on corneal allograft survival.

A



B

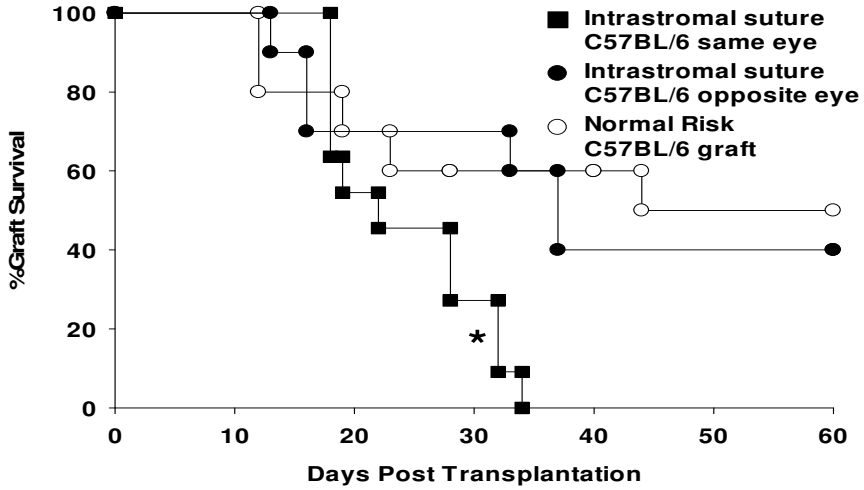


Figure 9. Intrastromal sutures do not produce sympathetic loss of immune privilege. A.) Clinical photo seven days after three 11.0 sutures were sewn through the stroma of a BALB/c cornea. Increased hemangiogenesis was observed in the corneal tissue. B.) BALB/c recipients that did not receive intrastromal sutures prior to receiving B6 corneal allografts (○; N = 10) rejected 50% of their B6 corneal allografts (MST = 34 days). Insertion of intrastromal sutures into the eyes resulted in 100% rejection of B6 corneal allografts placed into the sutured eye (MST = 18 days) (■; N = 10). Insertion of intrastromal sutures did not alter the 50% rejection rate or MST (33 days) of a B6 corneal allografts placed onto the contralateral eye (●; N = 10). *P <0.001

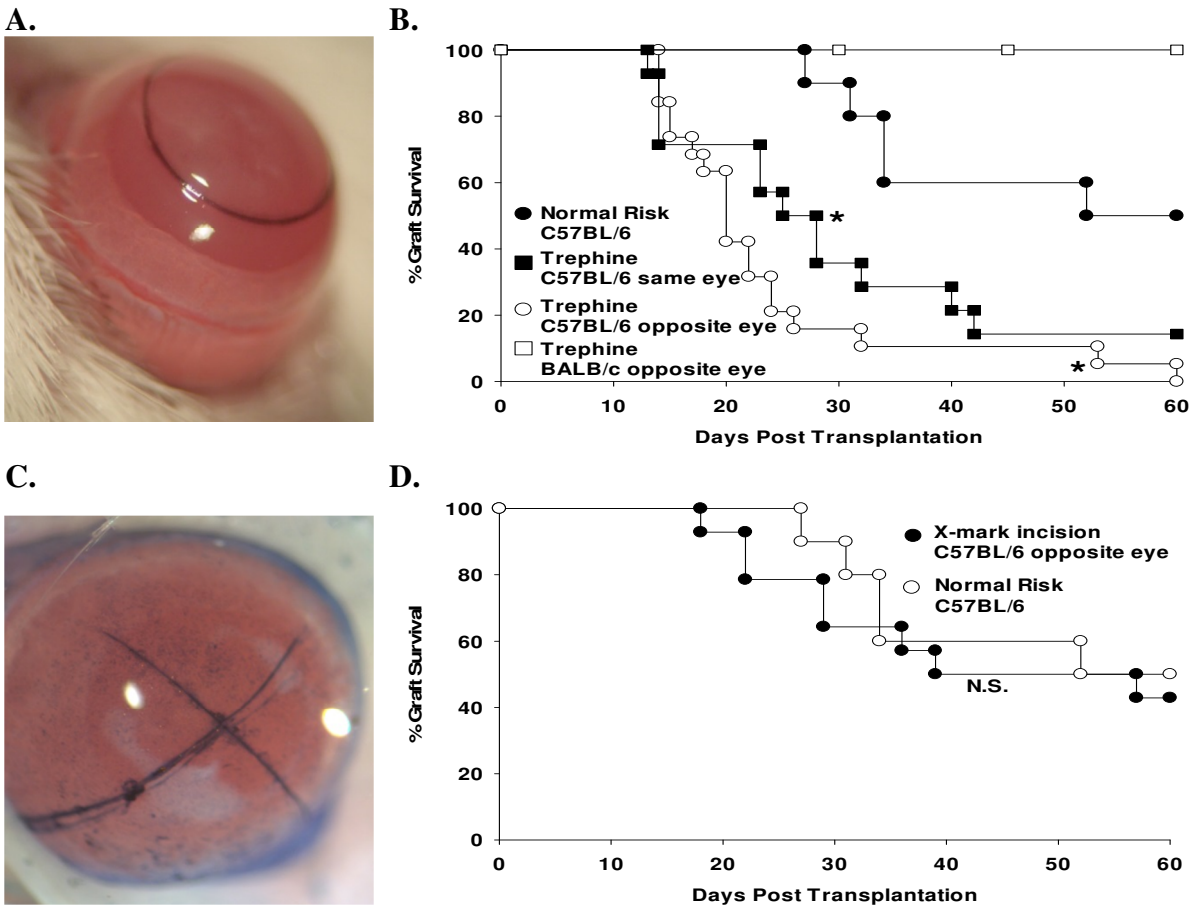


Figure 10. Circumferential incisions in one eye prevents the establishment of immune privilege in both eyes. A) Clinical photo of a 2.0 mm circumferential incision through the epithelium and stromal layers of the central cornea (stained with India ink). B.) BALB/c recipients that did not receive a corneal incision prior to receiving B6 corneal allografts (○; N = 10) rejected 50% of their B6 corneal allografts (MST = 35 days). BALB/c mice were treated by placing a 2.0 mm circumferential incision through the corneal epithelium and stroma of the central cornea prior to receiving B6 corneal allografts placed onto the same eye (◌; N = 19) or the contralateral unmanipulated eye (■; N = 18). Circumferential incisions did not induce rejection of syngeneic BALB/c corneal grafts (□; N = 12). C.) Clinical photo of an X-incision through the epithelium and stromal layers of the cornea (incision was stained with Evans blue). D) BALB/c mice that did were not treated with X-shaped corneal incisions prior to receiving B6 corneal allografts (N = 10, ○) had a 50% corneal graft rejection rate (MST = 35 days). Mice that were treated with X-shaped corneal incision in one eye 14 days prior to transplanting B6 corneal allografts into the contralateral eye had a rejection rate of 39% (MST = 36 days), which was not significantly different from untreated controls. (●). *P< 0.05 N.S = Not Significant.

Circumferential corneal surface incisions in one eye abolish immune privilege in the opposite eye and provoke the rejection of previously healthy corneal allografts.

Khodadoust, Donshik, and Williams observed a significant increased risk of rejection of first corneal allografts following the application of a second bilateral corneal allograft [218, 219]. However, studies by Buxton, Malbran, and Meyers, noted that although a previously healthy corneal transplant did undergo rejection after a second cornea was transplanted to the other eye, the incidence and tempo of rejection were not increased compared to unilateral corneal transplant recipients [220-222]. Since we observed that a circumferential incision of the corneal surface to one eye prevented the establishment of corneal allograft immune privilege in the other eye, we hypothesized that a circular incision to one eye would break established immune privilege in the other eyes. This disruption of immune privilege would render the corneal allograft vulnerable to immune destruction and result in the rejection of the accepted corneal allograft. To test this hypothesis, 30 days after transplantation of a B6 cornea, BALB/c recipients with a clear allograft received a circumferential incision in the contralateral eye, or were left unmanipulated. The unmanipulated recipients that did not received a circumferential incision to one eye, retained immune privilege and 100% of the corneal allografts survived. By contrast, a circumferential incision to the contralateral eye broke established immune privilege and resulted in a briefer MST (14 days) and 100% graft rejection (Figure 11).

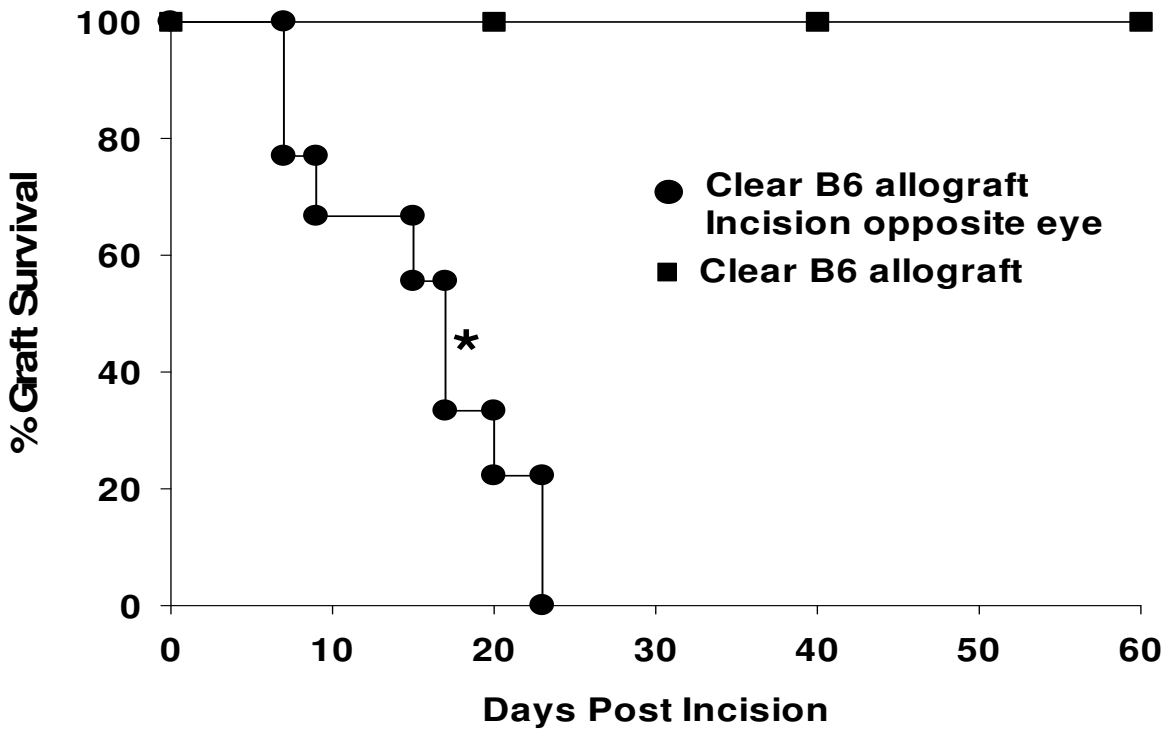


Figure 11. Circumferential corneal incisions in one eye breaks established immune privilege in the opposite eye and provoke graft rejection. BALB/c recipients with a clear corneal allograft received a circumferential incision in the opposite eye 30 days after the initial transplantation. Recipients with a clear corneal allograft and left untreated maintained (■) graft clarity and maintained the survival of 100% of their corneal allografts. A circumferential incision to the contralateral eyes of mice with previously clear, intact corneal allografts (●; N = 9) induced 100% rejection and an abbreviated MST (14 days). *P <0.001

Circumferential corneal incisions mediate acute and long-term loss of immune privilege in the contralateral eye.

A study by Tuft et. al. observed that patients with bilateral corneal allografts had a significantly increased risk of rejecting the first corneal transplant [223]. Moreover, the risk of rejection decreased as the time interval between the first graft and second graft increased [223]. We observed that recipients with a corneal allograft in one eye had a loss of immune privilege in the contralateral eye, even 60 days after the initial corneal transplant was performed (Figures 5 & 7). Accordingly, we wanted to determine if a circumferential corneal incision would abolish long-standing immune privilege in the other eye. B6 corneal allografts were transplanted 1, 14, 60 or 100 days after a circumferential incision was made in the opposite eyes of BALB/c mice. Circumferential incisions abolished immune privilege in the contralateral eyes when corneal allografts were transplanted as early as 1 day and as late as 60 days before corneal transplantation (One day rejection = 89%, MST = 22 days); (Fourteen days, rejection = 100%, MST = 20 days); (Sixty days, rejection = 90%, MST = 22). Moreover, a circumferential incision placed 100 days prior to corneal transplantation resulted in the same high incidence of graft rejection, albeit at a slower tempo (rejection = 87.5% MST = 34.5 days) (Figure 12). These results suggest that circumferential incisions produce an immediate and long-term loss of immune privilege in the other eye.

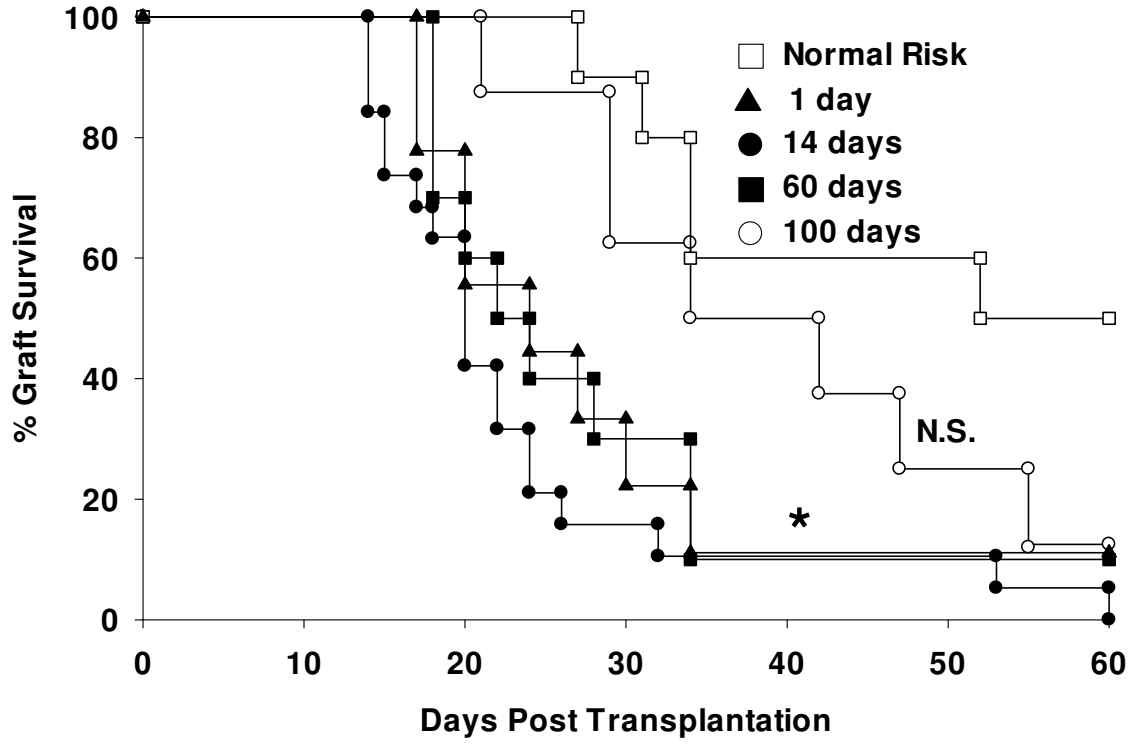


Figure 12. Circumferential incisions mediate an immediate and long term loss of immune privilege and induce corneal allograft rejection. Circumferential incisions were made in the central corneas of BALB/c mice 1, 60 or 100 days prior to receiving a B6 corneal allograft onto the contralateral eye. The incidence and tempo of rejection were identical in mice that received a circumferential incision one (▲; N = 9); 14 days (●; N = 19); or 60 days (■; N = 10) prior to transplantation and the allografts underwent rejection that was significantly swifter than unmanipulated corneal allograft recipients (□; N = 10). The incidence of rejection in mice that received a circumferential incision 100 days was significantly higher, but at the same tempo compared to normal risk recipients (○; N = 8). *P value < 0.05. N.S. = Not significant compared to normal risk.

Circumferential incisions disrupt corneal subbasal nerves.

The cornea is one of the most densely innervated tissues within the human body [7]. However, the innervation of the subbasal nerve plexus is severely decreased and disorganized in patients that undergo corneal transplantation, even 40 years after the surgery [18]. Experiments were performed to determine if a circumferential incision through the epithelium and stroma, but not the endothelium, disrupted the innervation processes within the corneal subbasal nerve plexus. Confocal microscopic examination of the subbasal nerve plexus in an unmanipulated eye revealed an even distribution of parallel β-Tubulin-III$^+$ nerve fibers innervating from the periphery into the central corneal tissue (Figure 13A). However, placing a circumferential incision through the epithelium and into 80% of the stroma disrupted the corneal innervation of the subbasal nerve plexus. The corneal nerves penetrated the corneal tissue from the periphery up to the incision line. However, there was no evidence of β-Tubulin III$^+$ corneal nerves in the central cornea beyond the incision line (Figure 13B). By contrast, X-shaped incisions produced minimal damage to the corneal nerves only at the point of the incision and did not affect corneal innervation in areas juxtaposed to the X-shaped incisions. The nerves in corneas subjected to X-shaped incisions penetrated the peripheral cornea up to the incision line, but were truncated at the incision. Most importantly, the X-shaped incision did not produce extensive destruction of the central corneal subbasal nerve plexus (Figure 13C). Thus, circumferential incisions, and not X-shaped incisions, of the corneal surface blunt innervation of the central cornea and elicit the destruction of the subbasal nerve plexus.

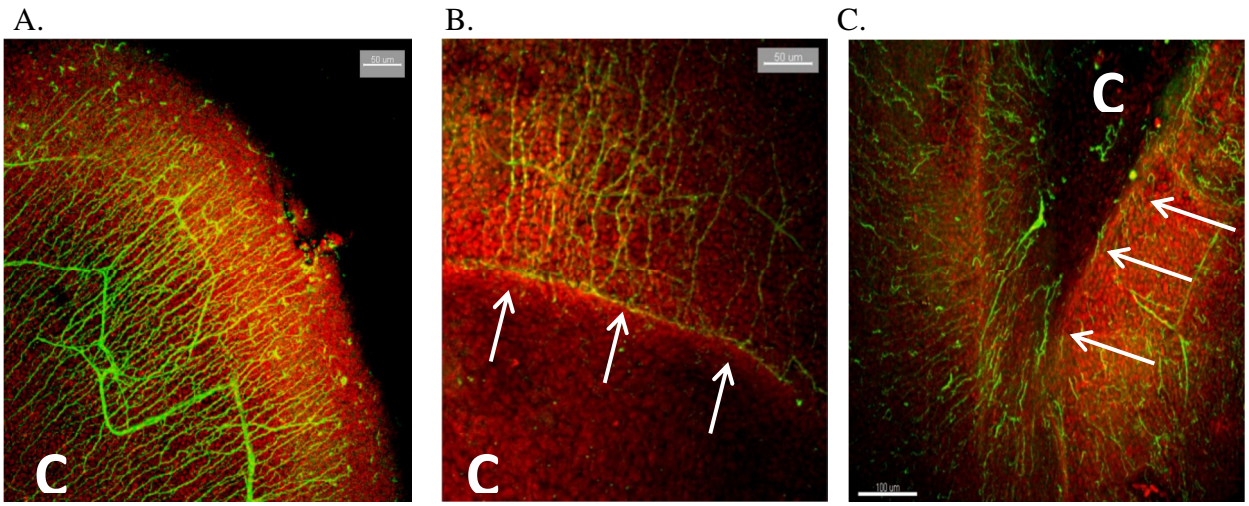


Figure 13. Circumferential incisions disrupt the innervation processes of the corneal subbasal nerve plexus. BALB/c corneas were collected from unmanipulated BALB/c eyes (A), Eyes that received a 2.0 mm circumferential incision (B), or 1.0 mm X-mark through the central cornea (C). The corneas were stained with propidium iodide (red) and β-tubulin-III (green). Arrows indicate site of incision. "C" indicates center of cornea.

Destruction of the corneal nerves in one eye alters the expression of immunoregulatory neuropeptides in the contralateral eye.

The neuropeptides, VIP, CGRP, somatostatin, and α-MSH have immunoregulatory roles that support ocular immune privilege by suppressing the innate and adaptive inflammatory reactions that occur within the AC of the eye [224], while SP breaks immune privilege by inhibiting the induction of immune tolerance [69]. Therefore, it was important to determine if the destruction of the corneal nerves seen with the circumferential incision in one eye led to the loss of immune privilege in the contralateral eye by altering the level of immunoregulatory neuropeptides. After the left eye of BALB/c hosts was circumferentially incised, the iris and corneal tissues were collected from the right eye at various time points, from 5 minutes to 14 days after the cornea was incised. The levels of the immunoregulatory neuropeptide mRNA and protein were analyzed by q-RT-PCR and EIA, respectively.

The contralateral eye showed a six-fold up-regulation of SP mRNA within 5 minutes of the circular incision procedure (Figure 14A), which correlated with a three-fold up-regulation of SP protein level in the contralateral corneal tissue. The level of SP protein within the contralateral eye peaked between 24 hours and 7 days with a 3-fold upregulation and a sustained 2-fold upregulation for up to 14 days (Figure 15A). CGRP protein level coincided with the level of SP, with a 2-fold upregulation at 24 hours, which remained elevated for 7 days. Moreover, the gene for α-MSH, Pomc, had a six-fold down-regulation of mRNA levels within one hour and retained a two-fold down-regulation through day 14 (Figure 14B). The diminished level of Pomc transcripts correlated with the 4-fold reduction in α-MSH protein levels in the contralateral eye by day 7. However, the level of α-MSH was restored to baseline levels by day 14 (Figure 15B). The circumferential incision reduced somatostatin protein levels to 86% of the baseline

level, which remained down-regulated for 7 days. However, somatostatin protein levels returned to baseline levels 14 days after the circumferential incision was created.

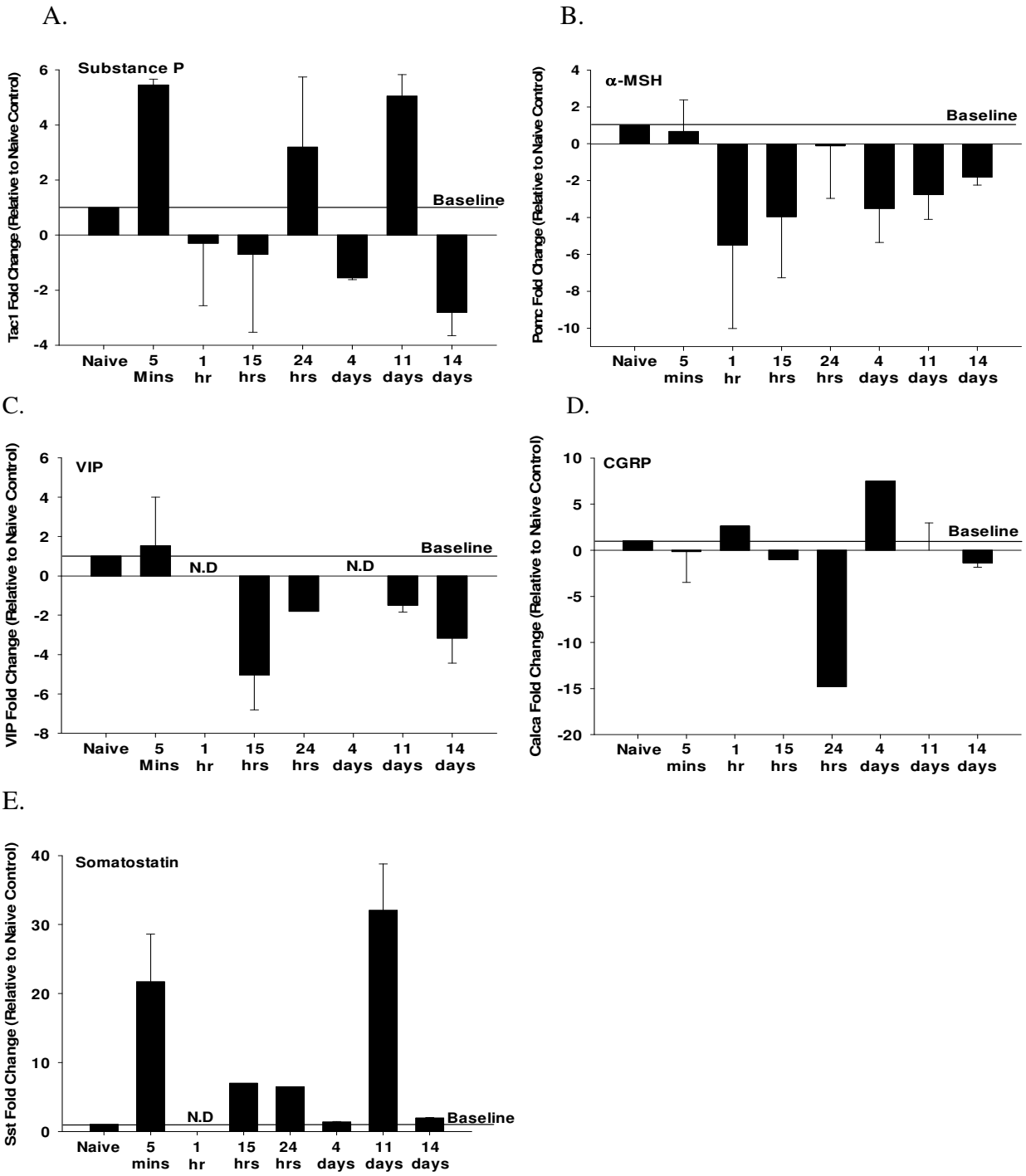


Figure 14. Destruction of corneal nerves alters the expression level of immunoregulatory neuropeptide mRNA in the contralateral eye. A circumferential incision was created in the left eye. At different time points after the incision was created, the anterior tissue (cornea and iris) from the right eyes of four mice was collected and pooled. Total mRNA was isolated from homogenized tissue and analyzed for SP, α-MSH, VIP, CGRP, and Sst by q-RT-PCR. Horizontal line represents baseline expression of each neuropeptide in eyes of normal mice. N.D. = Not Detected.

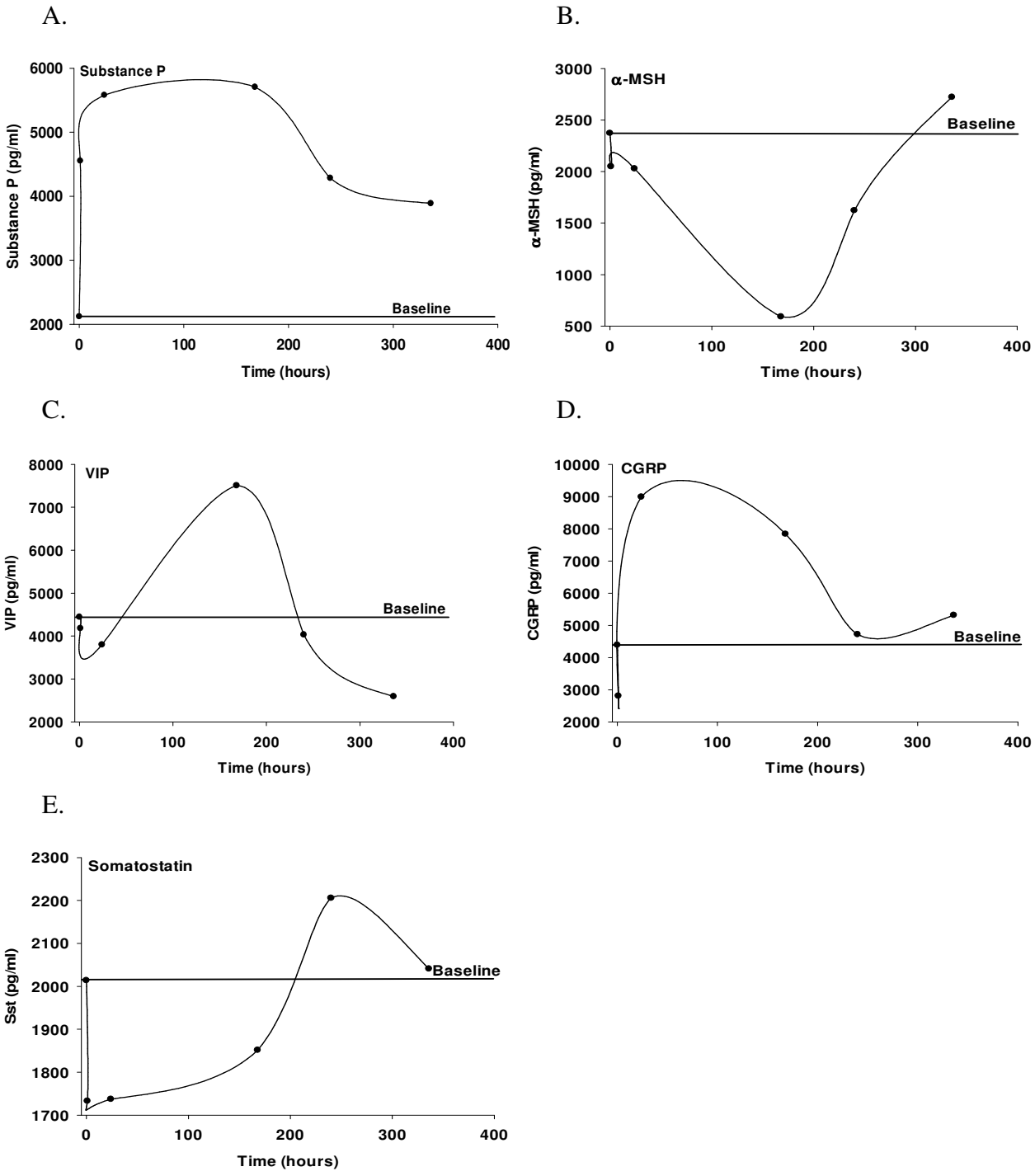
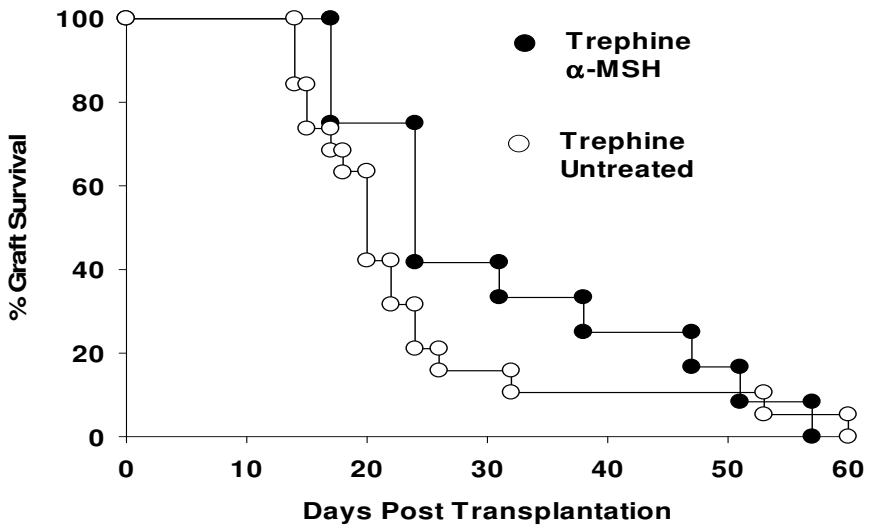


Figure 15. Destruction of corneal nerves alters the expression of immunoregulatory neuropeptide proteins in the contralateral eye. A circumferential incision was created in the left eye. At different time points after the incision was created, the anterior tissue (cornea and iris) from the right eyes five mice was collected and pooled. The tissue was homogenized by sonication and the supernatants were analyzed for SP, α-MSH, VIP, CGRP, and Sst by EIA. Horizontal line represents baseline expression of each neuropeptide in eyes of normal mice.

Exogenous α-MSH does not restore immune privilege in mice treated with circular incisions of the cornea prior to corneal transplantation.

Since the severing of the corneal nerves resulted in a significant decrease in the level of α-MSH in the contralateral eye, we wanted to determine if providing exogenous α-MSH would restore cornea allograft immune privilege. Previous studies showed that systemic administration of α-MSH alleviates the severity of experimental autoimmune uveitis [225]. We hypothesized that restoring α-MSH using a dosing scheme that was previously shown to alleviate autoimmune uveitis [225], would restore immune privilege and reduce corneal allograft rejection. Accordingly, BALB/c mice were treated with 2 μg of α-MSH given i.p. every 3 days from day -15 through day +1 and received a circumferential incision on the left eye on day -14 and a B6 corneal transplant on day 0. The results showed that providing exogenous α-MSH did not affect the incidence and tempo of rejection (rejection = 100%, MST = 20 days) compared to untreated recipients (rejection = 100%, MST = 20 days) (Figure 16A). Moreover, unmanipulated recipients treated with α-MSH did not show an enhancement of corneal allograft survival (rejection = 50%, MST = 40 days) compared to untreated recipients (rejection = 50%, MST = 46 days) (Figure 16B). This demonstrates that α-MSH cannot restore corneal transplantation immune privilege by enhancing the immunosuppressive environment.

A.



B.

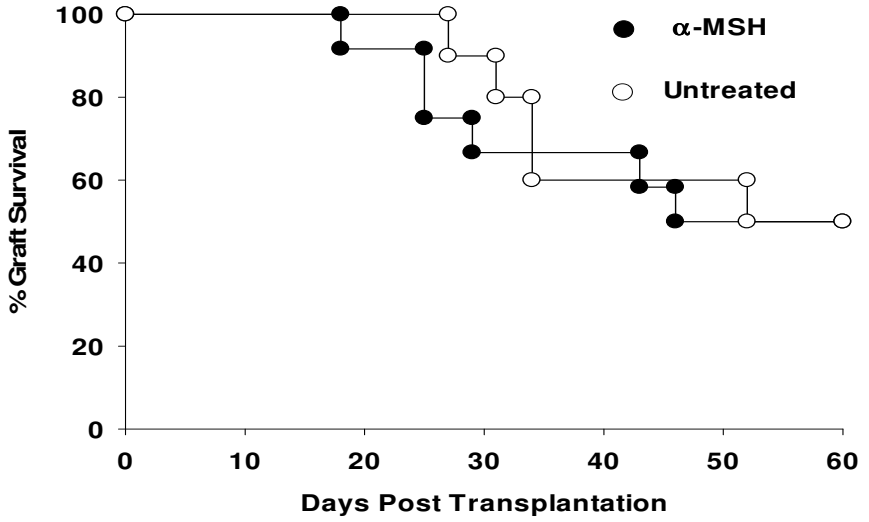


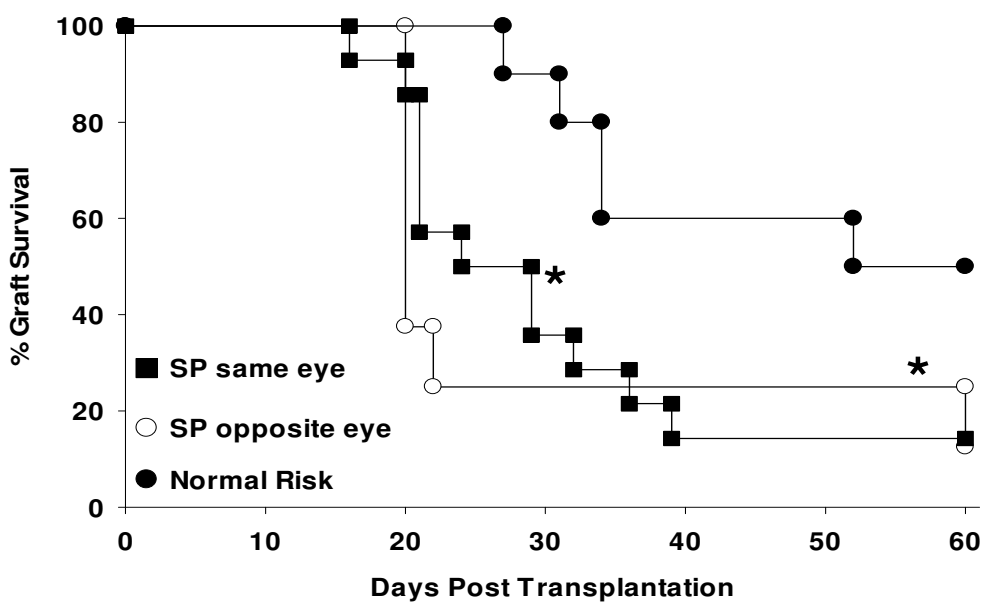
Figure 16. Exogenous α-MSH does not restore immune privilege of corneal allografts in mice receiving circumferential corneal incisions. A.) Circular incisions were made in the corneas of BALB/c mice 14 days prior to receiving B6 corneal allografts (day 0) on the contralateral eyes. Mice were either untreated or treated with 2 μg α-MSH given i.p. every 3 days from day -15 to day +1. Mice treated with α-MSH (●; N =12) had the same incidence and tempo of rejection as untreated mice (○; N = 19). B.) B6 corneal allografts were transplanted to BALB/c mice that were not treated with circular corneal incisions but were injected with 2 μg α-MSH i.p. every 3 days from day -1 to day +15. Mice treated with α-MSH (○; N = 12) had the same incidence and tempo of rejection as untreated normal risk corneal transplantation recipients (●; N = 10).P>0.05.

SP elicits an immediate and long-term loss of immune privilege in both eyes.

Since corneal incisions elevate SP production in the contralateral eye, which coincided with a sharp increase in the incidence and tempo of corneal allograft rejection in the contralateral eye, we hypothesized that administration of SP to mice not subjected to corneal incisions would abolish immune privilege and enhance graft rejection. Accordingly, BALB/c mice received a single 1.0 pg. subconjunctival injection of SP in the left one day prior to receiving a B6 corneal allograft on either the same eye or the contralateral eye. Mice treated with a single subconjunctival injection of SP given in either the same eye or the opposite eye had a significantly quicker tempo and higher rate of rejection of B6 corneal allografts (rejection = 85.75%, MST = 21 days and rejection = 87.5%, MST = 17 days respectively) compared to untreated control mice (Figure 17A). This exacerbation of graft failure was due to immune rejection and not due to non-specific toxic effects of SP, as subconjunctival injection of SP did not adversely affect the survival of syngeneic BALB/c corneal grafts (0% rejection) (Figure 17B). Since SP is a small peptide comprised of only 11 amino acids with a short in vivo half-life, we suspected that its effects might be transient. Accordingly, a single injection of 1.0 pg. of SP was injected subconjunctivally into the left eyes of BALB/c mice either 60 days or 100 days prior to the application of B6 corneal allografts. The results showed that the untoward effects of subconjunctival injection of SP dissipated over time (Figure 18). Corneal allografts transplanted either 60 days or 100 days after subconjunctival injection of SP displayed MSTs that were not significantly different from untreated controls: 60 day group: rejection = 62.5%; MST = 48 days; 100 day group: rejection = 50%; MST = 51 days ; and control group: rejection = 50%; MST = 46 days (Figure 18).

The finding that subonconjunctival injection of SP into one eye resulted in exacerbation of corneal allograft rejection in the other, untreated eye suggested that the effects of SP in the abolition of immune privilege were systemic. We tested this hypothesis by injecting 1.0 pg. of SP i.v. either one day or 100 days prior to the application of B6 corneal allografts. The administration of a single injection of SP either one day or 100 days prior to transplantation resulted in a high incidence of graft rejection (rejection = 100%; MST = 28 days and rejection = 100%; MST = 28 days respectively), (Figure 19). These results demonstrate that SP can abolish immune privilege and provoke a high incidence of graft rejection. The administration of SP, either into the subconjunctival milieu or systemically, results in an immediate and long-lasting loss of immune privilege. However, the long-lasting loss of immune privilege was found only when SP was administered systemically.

A.



B.

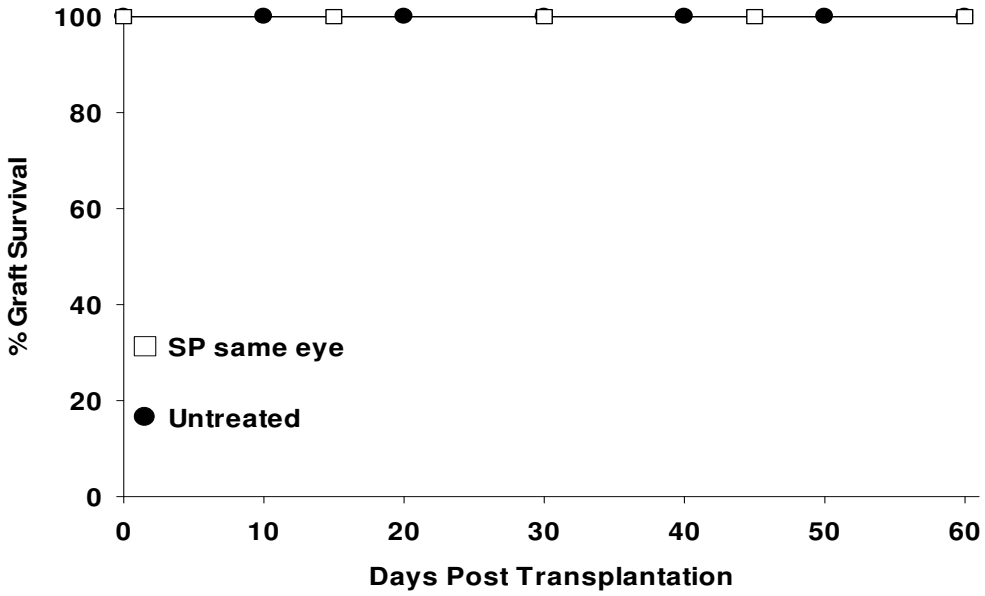


Figure 17. Local SP promotes corneal allograft rejection. A.) One pg of SP was injected subconjunctivally into the left eyes of BALB/c mice one day prior to the application of B6 corneal allografts onto either the left eye or the contralateral right eye. SP treated mice had a higher incidence of graft rejection when SP was injected into either the grafted eye (■; N = 14) or the contralateral eye (◌; N = 8) compared to untreated mice (●; N = 10). B.) BALB/c recipients received 1 pg SP in the subconjunctiva of the left eye one day prior to receiving a syngeneic BALB/c corneal graft onto the same eye (□; N = 9). The injection of SP did not elicit corneal graft failure as all of the syngeneic grafts remained clear in mice treated with SP.*P = 0.02

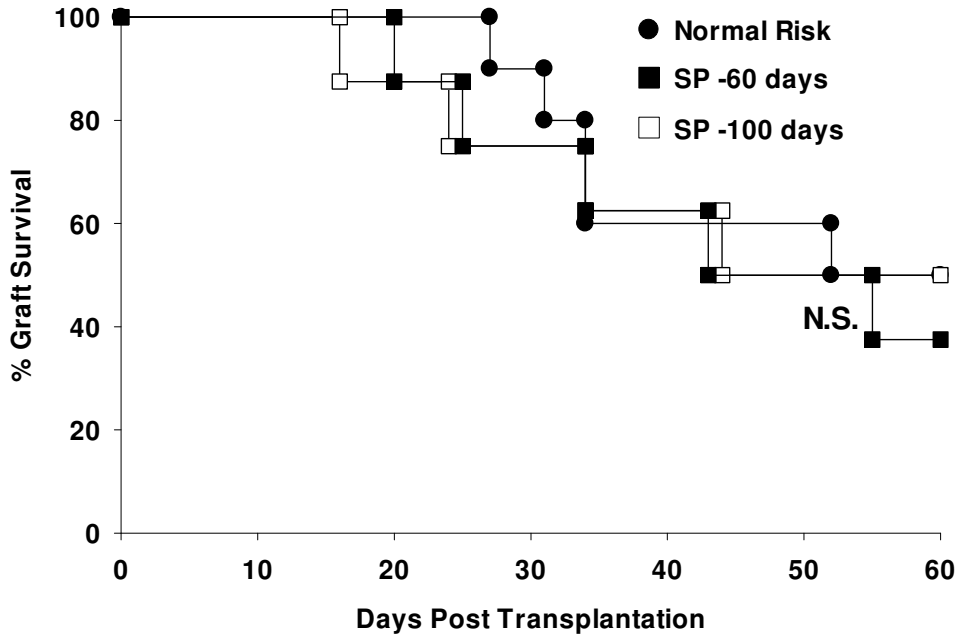


Figure 18. Administration of SP to the ocular environment does not abolish immune privilege over the long-term. One pg of SP was injected subconjunctivally into the left eyes of BALB/c mice either 60 or 100 days prior to the application of B6 corneal allografts onto the left eyes. Corneal allografts transplanted to mice that received a single bolus injection of one pg SP 60 days (■; N = 8) or 100 days (□; N = 8) had the same tempo and incidence of rejection compared to corneal allografts transplanted to untreated hosts (●; N = 8). *P value < 0.05. N.S. = Not significant compared to Normal Risk.

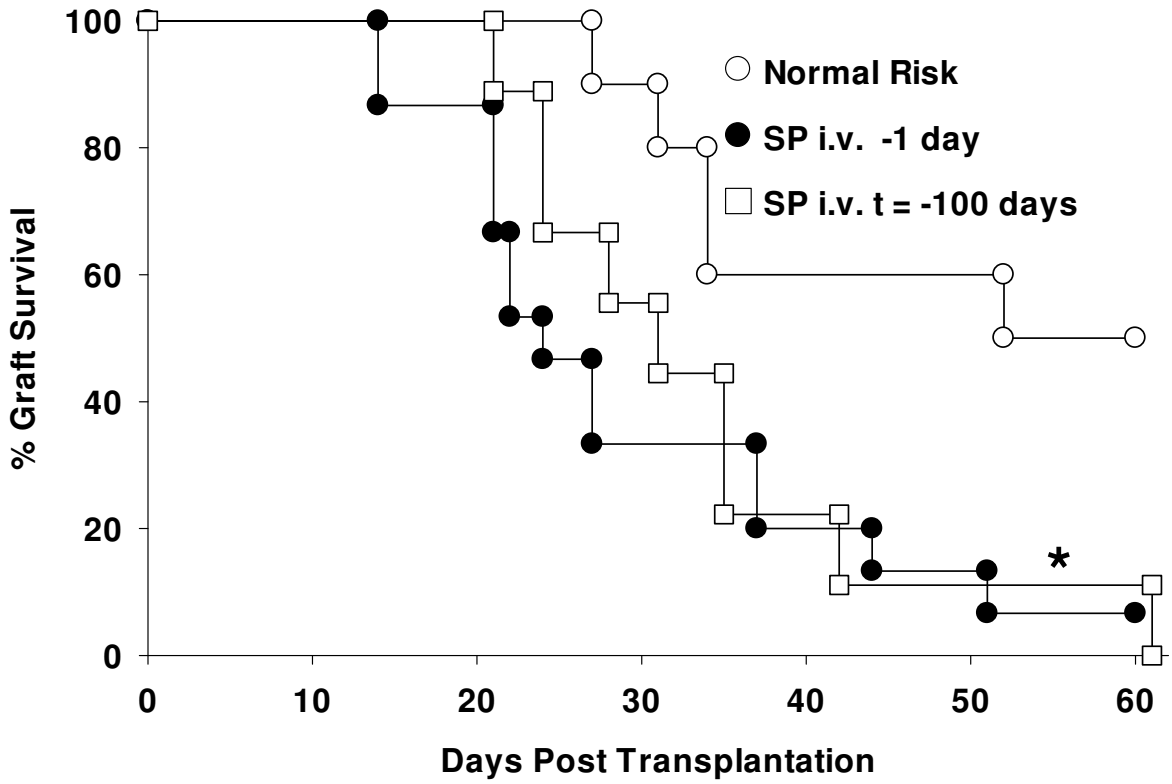


Figure 19. Systemic administration of SP constantly abrogates immune privilege. One pg of SP was injected i.v. into BALB/c either one day or 100 days prior to the application of B6 corneal allografts. SP injected one day (●; N = 15) or 100 days (□; N= 9) prior to corneal transplanted compared to naïve corneal allograft recipients (○; N = 10). *P = 0.02

Blocking SP restores immune privilege in mice treated with corneal incisions prior to corneal transplantation.

The results to this point showed that severing corneal nerves in one eye results in a steep increase in the level of SP in the contralateral eye and leads to the exacerbation of corneal allograft rejection. These findings also indicated that the exacerbation of corneal allograft rejection that occurs in mice subjected to corneal incisions could be recapitulated by administering exogenous SP to naïve mice. This prompted us to determine if blocking the activity of SP would restore immune privilege and enhance corneal allograft survival in mice subjected to corneal incisions. To address this, a modified analogue of SP, Spantide II, was injected the day prior to making circumferential corneal incisions and every day thereafter to block NK-1R, the receptor for SP. The corneal nerves were severed by trephining the left eye, and 14 days later, B6 corneal allografts were transplanted to the right eye. Systemic blockade of the receptor for SP with Spantide II reduced the incidence of graft rejection (rejection = 68%; MST = 33.5 days) compared to untreated recipients (rejection = 100%; MST = 18 days) (Figure 20A).

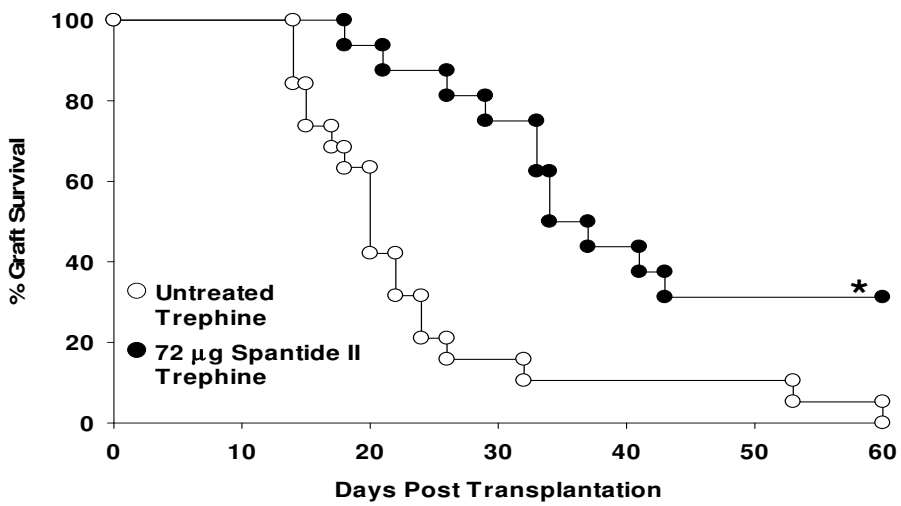
Since severing the corneal nerves in one eye produces a sustained loss of immune privilege for corneal allografts placed in the contralateral eye, we wanted to determine if the prolonged loss of immune privilege was permanent or could be reversed. Accordingly, circumferential corneal incisions were made in the left eyes of BALB/c mice sixty days prior to transplanting B6 corneal allografts to the right eyes. To address the hypothesis that the prolonged loss of immune privilege could be reversed by blocking SP activity, circular incisions were made in the left eyes of BALB/c mice. 72 μg of Spantide II was administered i.p. one day prior to the application of B6 corneal allografts to the right eyes and continued daily throughout

the course of the experiments. Blocking SP through the administration of Spantide II restored immune privilege and significantly reduced the incidence and tempo of rejection (rejection = 37.5%; MST = 60 days) compared to untreated controls (rejection = 90%; MST = 22 days) (Figure 20B).

The restoration of immune privilege through the administration of Spantide II in mice subjected to circular corneal incisions prompted us to test if Spantide II treatment would enhance graft survival in mice not subjected to corneal incisions prior to the application of corneal allografts. Accordingly, BALB/c recipients were treated with a low dose (36 μg) or high dose (72 μg) of Spantide II given i.p. one day prior to the application of B6 corneal allografts and treatment was continued everyday afterwards. The previous results showed that blocking SP reversed the deleterious effects of circular corneal incisions, however blockade of SP with either dose of Spantide II did not enhance corneal allograft acceptance under the routine conditions for corneal transplantation in which corneal incisions are not made prior to orthotopic corneal transplantation; 42.85% rejection and MST = 60 days in 36 μg Spantide II-treated hosts compared to 50% rejection and MST = 46 days in untreated graft hosts (Figure 21). These results suggest that the initial burst of SP caused by the corneal nerve incision is one of the root causes of the loss of corneal allograft immune privilege in the contralateral eye. Blocking SP signaling, either prior to the corneal nerve laceration or after the initial burst of SP subsides, can restore corneal allograft immune privilege. However, the effect of SP is specific for recipients that received a corneal nerve incision in one eye prior to receiving a corneal allograft in the other eye as blocking SP restores the immune privilege back to basal levels. Blocking SP does not enhance corneal allograft survival in initial corneal allograft recipients, as mice that had intact

corneal nerves did not have an enhancement of corneal allograft survival following treatment with Spantide II.

A.



B.

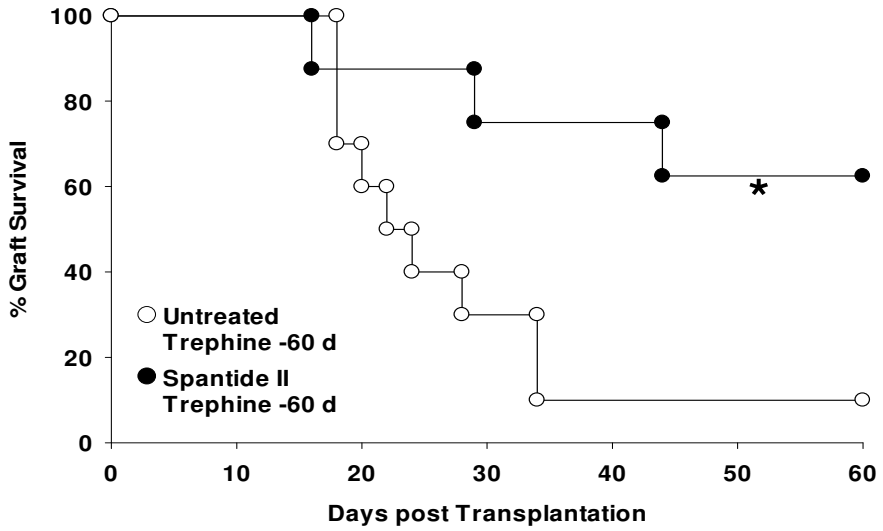


Figure 20. Blocking SP activity in mice with corneal incisions restores immune privilege. Corneal allograft survival in BALB/c recipients treated with Spantide II after receiving a circumferential incision in the contralateral eye. A.) Circumferential incisons were placed in the corneas of the left eyes of BALB/c fourteen days prior to the application of B6 corneal allografts. Mice were treated with i.p. injections of Spantide II (72 µg/injection) beginning one day prior to the placement of corneal incisions and each day thereafter until day 60 or the day of graft rejection, whichever occurred first. Corneal allografts underwent rejection in 100% of the untreated mice (○; N = 19; MST = 18 days). Spantide II treatment increased corneal allografts survival rate to 32% and prolonged the MST to 33 days (●; N= 16). B.) BALB/c recipients received a circumferential incision to one eye 60 days prior to receiving a B6 corneal transplant onto the contralateral eye. Mice were treated daily with i.p. injections of 72 µg of Spantide II beginning one day prior to corneal transplantation and continuing until the day of rejection or until day 60, whichever occurred first. Corneal allografts underwent rejection in 90% of the untreated controls mice (○; N = 10; MST =19 days). Spantide II treatment increased corneal allografts survival rate to 62.5% and prolonged the MST to 23 days (●; N= 8). *P = 0.017

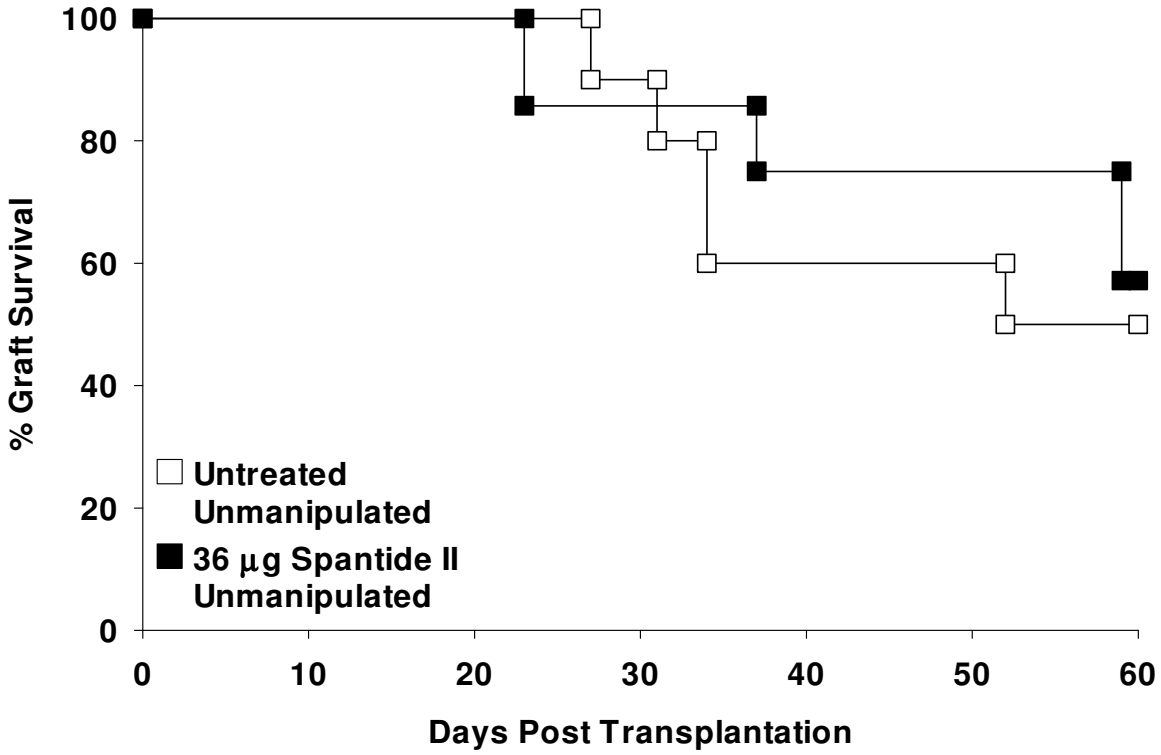
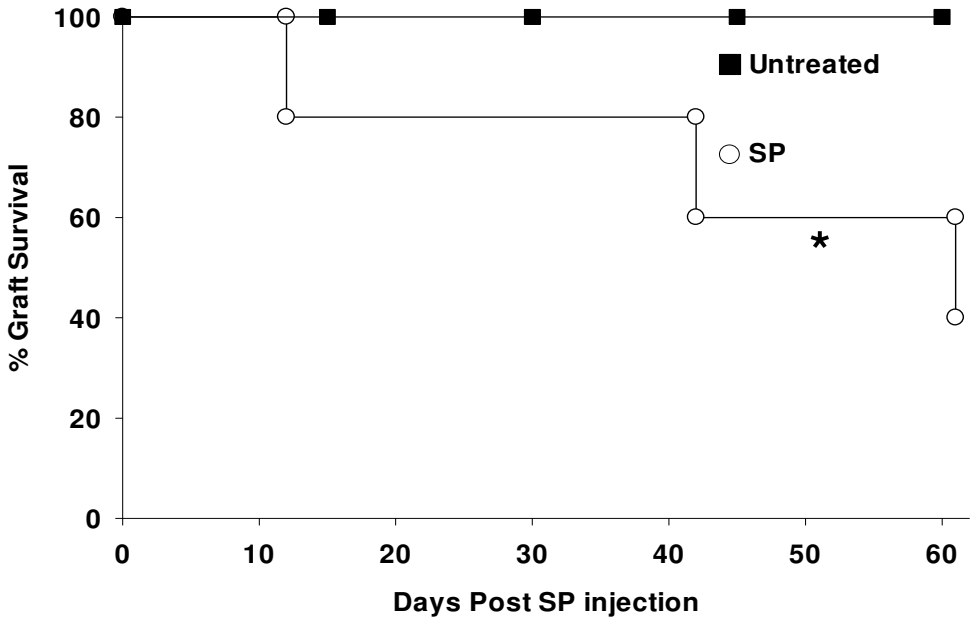


Figure 21. Blocking SP activity does not enhance corneal allograft survival in normal risk recipients. B6 corneal allografts were transplanted to BALB/c mice that were either untreated or treated daily with i.p. injections of Spantide II (36 µg/day) beginning one day prior to corneal transplantation and daily until day 60. Mice treated with Spantide II (■; N = 8) had the same incidence of rejection as untreated corneal allograft recipients (□; N = 10). P> 0.05

Circumferential corneal incisions do not abrogate established immune privilege in the initial eye via SP

Since SP inhibited the generation of immune privilege, we hypothesized that SP also breaks existing immune privilege and leads to the rejection of an established corneal allograft. To address this hypothesis, mice with corneal allografts that had remained clear for 30 days received one pg of SP injected into the conjunctiva of the transplanted eye and the fate of the existing corneal transplant was followed. The results showed that a single subconjunctival injection of exogenous SP broke immune privilege and elicited rejection of the established corneal allografts (rejection = 40%; MST = 60 days) (Figure 22A). Since SP can abrogate existing immune privilege, we hypothesized the corneal nerve laceration to one eye would have the same effect and lead to the rejection of established corneal allografts. To address this hypothesis, BALB/c mice with B6 corneal allografts that had been in place and clear for 30 days were treated with i.p. injections of Spantide II on day 30 post-transplantation. One day later, circular incisions were placed into the corneas of the opposite eye. Daily Spantide II treatment was continued for another 30 days or until the corneal allografts underwent rejection. Blocking SP did not prevent the rejection of corneal allograft in hosts subjected to corneal incisions. That is, mice treated with Spantide II had the same incidence and tempo of rejection as untreated controls (rejection =100%; MST =15 days and rejection = 100%; MST = 14 days respectively; $P>0.05$) (Figure 22B).

A.



B.

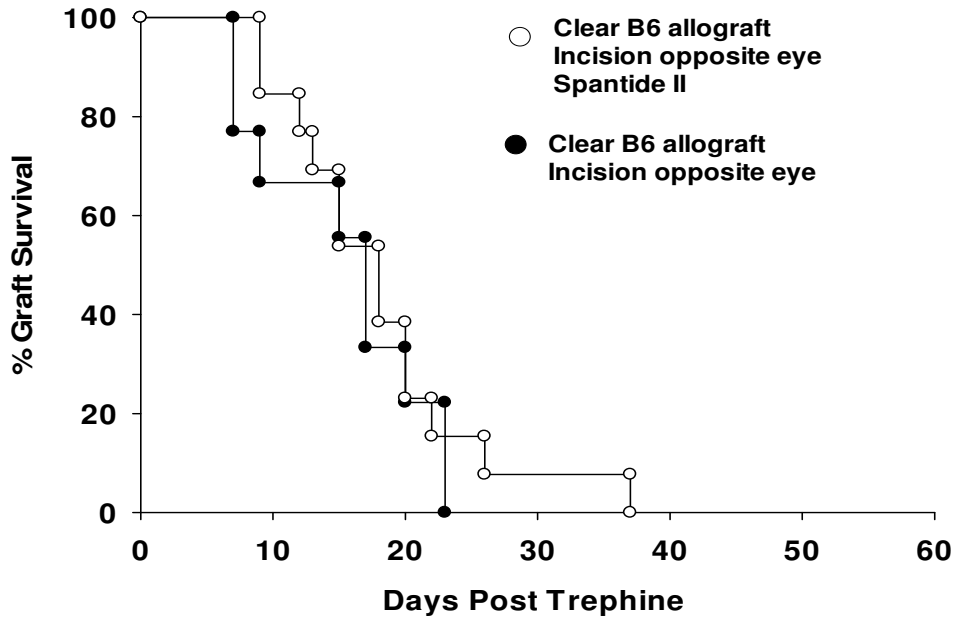


Figure 22. Corneal nerve incision reverses established immune privilege. A.) BALB/c mice with clear B6 corneal allografts after 30 days, either received one pg of SP injected into the subconjunctiva of the grafted eye or were left untreated. Mice with clear corneal allografts and left untreated (■) maintained graft clarity and accepted 100% of their corneal allografts for 60 days. Injections of SP (○; N = 6) into the conjunctivae of the clear corneal allografts stimulated rejection in 40% of the mice. B.) BALB/c hosts that harbored clear B6 corneal allografts for 30 days were treated with circumferential incisions placed into the corneas of the contralateral eyes. Untreated mice (●; N = 9) or mice treated with 72 ug Spantide II i.p. (○; N = 13) daily had 100% graft rejection with MSTs of 14 days and 15 days, respectively.

Corneal nerve laceration does not render $CD4^+$ T effector cells resistant to suppression by $CD4^+CD25^+$ Tregs

As stated earlier, corneal transplant survival requires the induction of $CD4^+CD25^+$ Tregs to maintain immune privilege by suppressing the alloimmune response [158, 161]. Depleting Tregs with anti-CD25 antibody treatment increases corneal allograft rejection from 50% to 100% [141]. Moreover, adoptive transfer of $CD4^+CD25^+$ Tregs into naïve BALB/c allograft recipients prior to placing a B6 corneal allograft increases corneal allograft survival [161]. Our next aim was to determine if the ablation of corneal nerves alters the tolerogenic interactions between Tregs and T effector cells and results in corneal allograft rejection.

BALB/c mice with on-going allergic conjunctivitis develop T effector cells that become resistant toward Treg cell-mediated suppression and reject 90 to 100% of their B6 corneal allografts [148, 149]. We wanted to determine if the loss of immune privilege in mice subjected to circumferential corneal incisions is due to the development of T effector cells that resist suppression by corneal allograft-induced Tregs in a manner similar to what occurs in mice with allergic conjunctivitis. A local adoptive transfer (LAT) assay was employed to determine if the T effector cells isolated from mice treated with circumferential corneal incisions fourteen days prior to receiving B6 corneal allografts onto the contralateral eye were resistant to suppression mediated by corneal allograft-induced Tregs. In this *in vivo* suppression assay, Tregs from graft acceptor mice were mixed with BALB/c APCs pulsed with B6 alloantigens and T effector cells from BALB/c mice that had recently rejected their B6 corneal allografts or from BALB/c mice that had been treated with circumferential corneal incisions prior to the application of B6 corneal allografts and as a result, rejected 100% of their B6 corneal allografts. The cell suspensions were then injected into the ears of naïve mice and the DTH responses were measured 24 hrs. later. T

effector cells isolated from mice that had either recently rejected B6 corneal allografts or mice that had been treated with circumferential corneal incisions produced robust DTH responses. Moreover, Tregs collected from mice with surviving corneal allografts suppressed the DTH responses of T effector cells from both rejector mice not treated with corneal incisions and rejector mice that had been pre-treated with corneal incision (Figure 23). These results suggest the exacerbation of corneal allograft rejection in mice that occurs following circumferential corneal incision is not due to effector T cells becoming resistant to the suppressive effects of T regulatory cells.

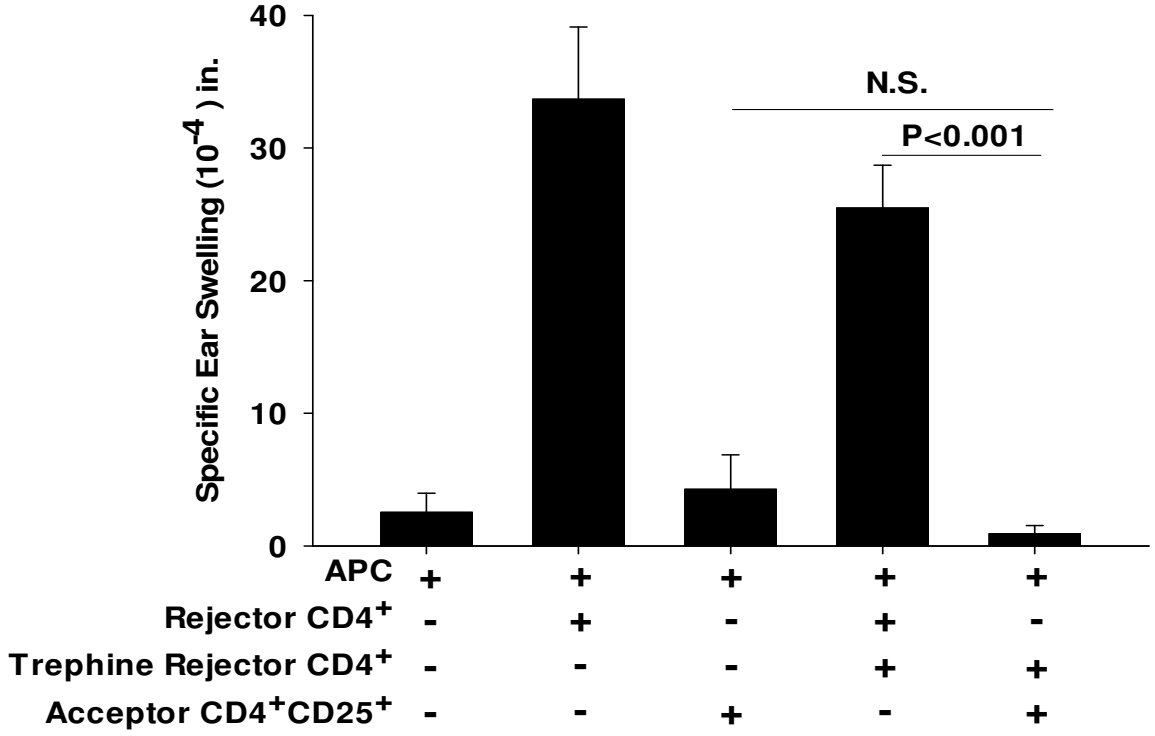


Figure 23. Circumferential corneal incisions do not render $CD4^+$ T effector cells resistant to $CD4^+CD25^+$ Treg suppression. Corneal allograft-induced $CD4^+CD25^+$ T cells were mixed with $CD4^+$ T cells isolated from unmanipulated BALB/c mice or mice subjected to circumferential corneal incisions 14 days prior to receiving B6 corneal allografts placed into the opposite eye and APCs pulsed with B6 alloantigen and tested in a conventional LAT assay for in vivo suppression of DTH. N.S. = Not Significant; N = 5. These experiments were conducted twice with similar results.

Circumferential incisions inhibit the generation of $CD4^+CD25^+$ Tregs

A second hypothesis to account for the exacerbation of corneal allograft rejection in mice treated with circumferential corneal incisions posits that corneal incisions disrupt the generation or function of $CD4^+CD25^+$ Tregs. To address this hypothesis, we tested if the ablation of the corneal nerves inhibited the generation of $CD4^+CD25^+$ Tregs. T effector cells were isolated from either untreated BALB/c mice that had recently rejected their B6 corneal allografts or mice that had been treated with corneal incisions and had recently rejected B6 corneal allografts. The effector T cells from each group of corneal graft rejector mice were mixed with BALB/c APC that had been pulsed with B6 alloantigens and $CD4^+CD25^+$ Tregs from graft acceptors. The cell suspensions were injected into the ears of naïve mice and DTH responses were assessed 24 hrs. later. Tregs from graft acceptor mice significantly inhibited the DTH ear swelling responses mediated by T cells from rejector mice. However, the $CD4^+CD25^+$ putative Tregs from normal risk rejectors or corneal incision rejectors did not suppress DTH responses (Figure 24). These results indicate that circumferential corneal incisions prevent the generation of Tregs that suppress allospecific DTH responses. This is consistent with previous observations indicating that corneal allograft survival is intimately associated with suppression of donor-specific DTH responses [226].

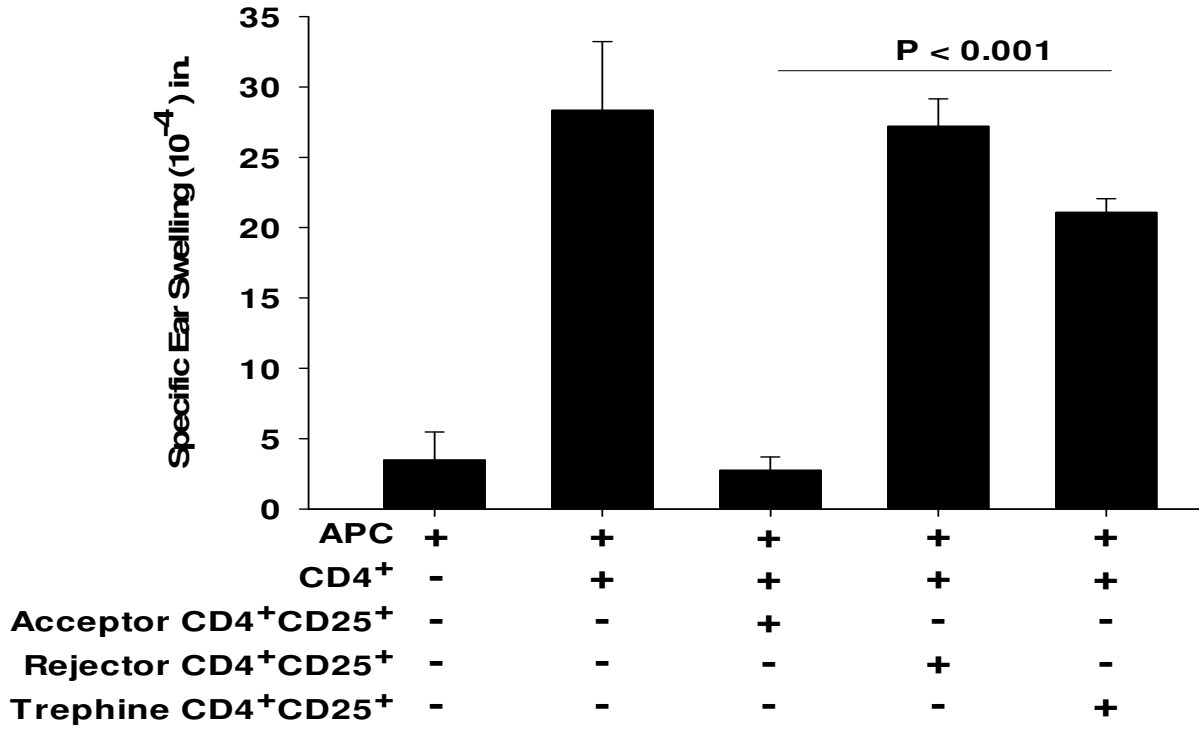


Figure 24. Severing corneal nerves prevents the generation of corneal allograft-induced Tregs. The suppressive properties of $CD4^+CD25^+$ T cells were tested in a LAT assay. Effector cells were isolated from BALB/c mice that had recently rejected B6 corneal allografts. $CD4^+CD25^+$ putative Tregs were isolated from either unmanipulated BALB/c mice that had accepted B6 corneal allografts, unmanipulated BALB/c mice that had recently rejected B6 corneal allografts, or BALB/c mice subjected to circumferential corneal incisions 14 days prior to receiving B6 corneal allografts. Normal BALB/c APCs were pulsed with B6 alloantigens. All three cell populations were mixed and injected into the ears of naïve BALB/c mice and DTH responses were assessed by measuring specific ear swelling 24 hrs. later. N = 5. These experiments were conducted twice with similar results.

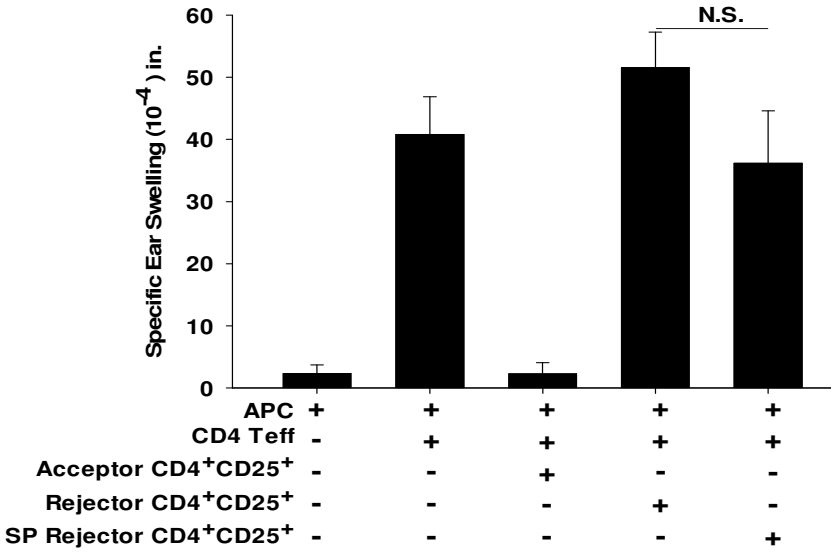
SP inhibits the generation and function of $CD4^+CD25^+$ Tregs

We hypothesized that the release of SP in response to corneal incisions prevented the generation of Tregs in mice that received corneal allografts. Accordingly, we determined if the administration of SP prior to corneal transplantation would inhibit the induction of functionally suppressive $CD4^+CD25^+$ T cells. $CD4^+$ effector T cells from normal risk graft rejectors and BALB/c APC pulsed with B6 alloantigens were mixed with $CD4^+CD25^+$ putative Tregs from either graft acceptors, normal risk graft rejectors, or SP injected graft rejectors and injected into the ears of naïve BALB/c mice. Twenty-four hrs. later DTH responses were measured. $CD4^+CD25^+$ Tregs from graft acceptor mice reduced the ear swelling DTH responses thereby confirming their suppressive properties. However, $CD4^+CD25^+$ putative Tregs from normal risk rejector mice or from SP-injected rejector mice did not suppress DTH responses (Figure 25A). These results suggest that SP elaborated in response to corneal incisions prevents the induction of Tregs that normally occurs in 50% of BALB/c mice that receive B6 corneal allografts and maintain long-term survival of their B6 corneal allografts. This in turn, robs the corneal transplant of its immune privilege.

The previous results suggest that SP can inhibit the induction of tolerance by preventing $CD4^+CD25^+$ Tregs from attaining their suppressive function. Next, we wanted to determine if SP could directly abolish otherwise functional Tregs from mediating suppression. To address this hypothesis, $CD4^+$ T effector cells from normal risk rejectors, BALB/c APCs pulsed with B6 alloantigens, and Tregs from graft acceptors were co-injected into the ears of naïve BALB/c mice, in the presence or absence of 1 pg. exogenous SP. The addition of SP to APCs and $CD4^+$ T effector cells did not enhance DTH responses compared to stimulated $CD4^+$ T effector cells without the addition of exogenous SP. However, the presence of SP abrogated the suppressive

function of Tregs that would otherwise inhibit DTH responses (Figure 25B). These results suggest that SP can break established immune tolerance by inhibiting the suppressive function of previously functional $CD4^{+}CD25^{+}$ Tregs.

A.



B.

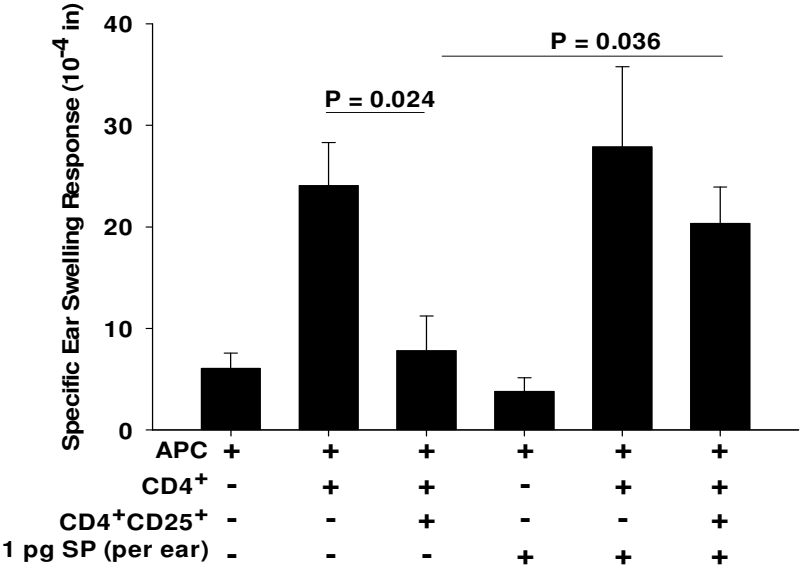


Figure 25. SP prevents the generation and function of Tregs induced by corneal allografts. A.) Corneal allograft-induced $CD4^+$ T cells were mixed with APCs pulsed with B6 alloantigen and $CD4^+CD25^+$ T cells isolated from unmanipulated BALB/c mice or BALB/c mice that received one pg. of SP injected into the conjunctivae one day prior to the transplantation of a B6 corneal allograft. The ability of $CD4^+CD25^+$ Treg to mediate their suppressive function was tested in a conventional LAT assay. B.) Corneal allograft-induced $CD4^+$ T cells were mixed with $CD4^+CD25^+$ T cells isolated from unmanipulated BALB/c mice that accepted B6 corneal allografts and APCs pulsed with B6 alloantigen in the presence or absence of 1 pg. of SP. N.S. = Not Significant; N = 5. Experiments were conducted in 2 independent experiments with similar results.

Circumferential incisions do not alter the expression of molecules associated with the suppressive function of corneal allograft-induced $CD4^+CD25^+$ Tregs

The optimal suppressive function of corneal allograft-induced Tregs requires the expression of m-TGF-β, CTLA-4, and GITR [158]. Thus, we suspected that circumferential incisions altered $CD4^+CD25^+$ T cell suppressive function by reducing the expression of one or more of these suppressive molecules. Thus, $CD4^+CD25^+$ T cells were isolated from normal risk acceptors, normal risk rejectors, or corneal incision rejectors were stimulated with anti-CD3ε and anti-CD28. The protein and mRNA levels of FoxP3, CTLA-4, GITR, and m-TGF-β were evaluated by flow cytometry (Figure 26) and q-RT-PCR (Figure 27), respectively. $CD4^+CD25^+$ T cells collected from normal risk rejectors and corneal incision rejectors showed no significant decrease in the expression of any of the suppressive molecules at either the protein or the mRNA level compared to normal risk acceptors. This suggests the loss of suppressive activity by Tregs isolated from corneal incision rejector mice was not due to a decreased expression of FoxP3, CTLA-4, GITR, of m-TGF-β. The cause of the defective Treg activity that occurs in mice treated with circumferential corneal incisions remains unresolved.

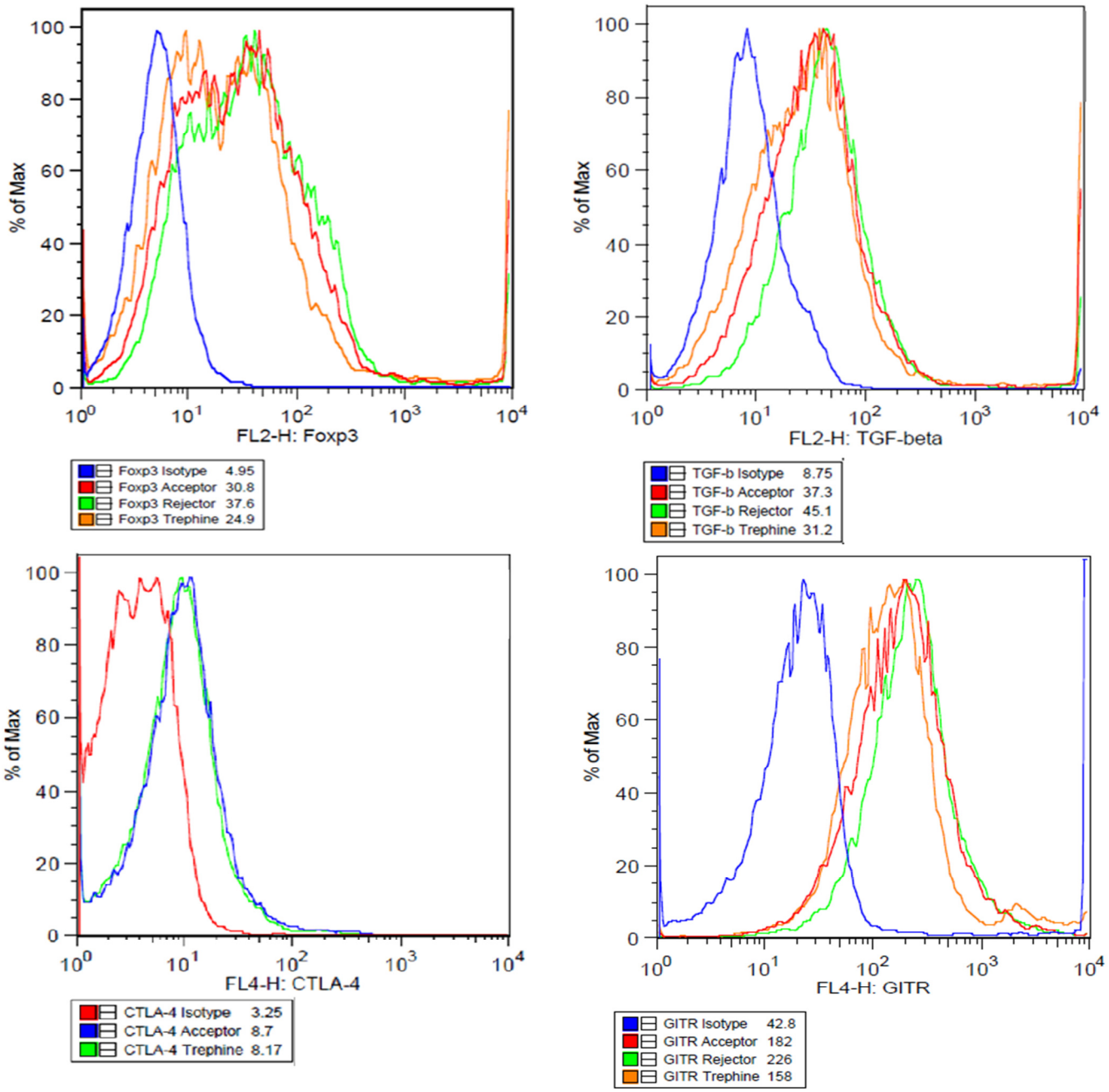


Figure 26. Disruption of the corneal nerves does not affect the expression of suppressive molecules on $CD4^+CD25^+$ T cells. The expression of A.) FoxP3, B.) TGF-β1, C.) CTLA-4, and D.) GITR was assessed on $CD4^+CD25^+$ T cells 72 hours following anti-CD3ε and anti-CD28 stimulation. This experiment was performed 4 separate times with similar results.

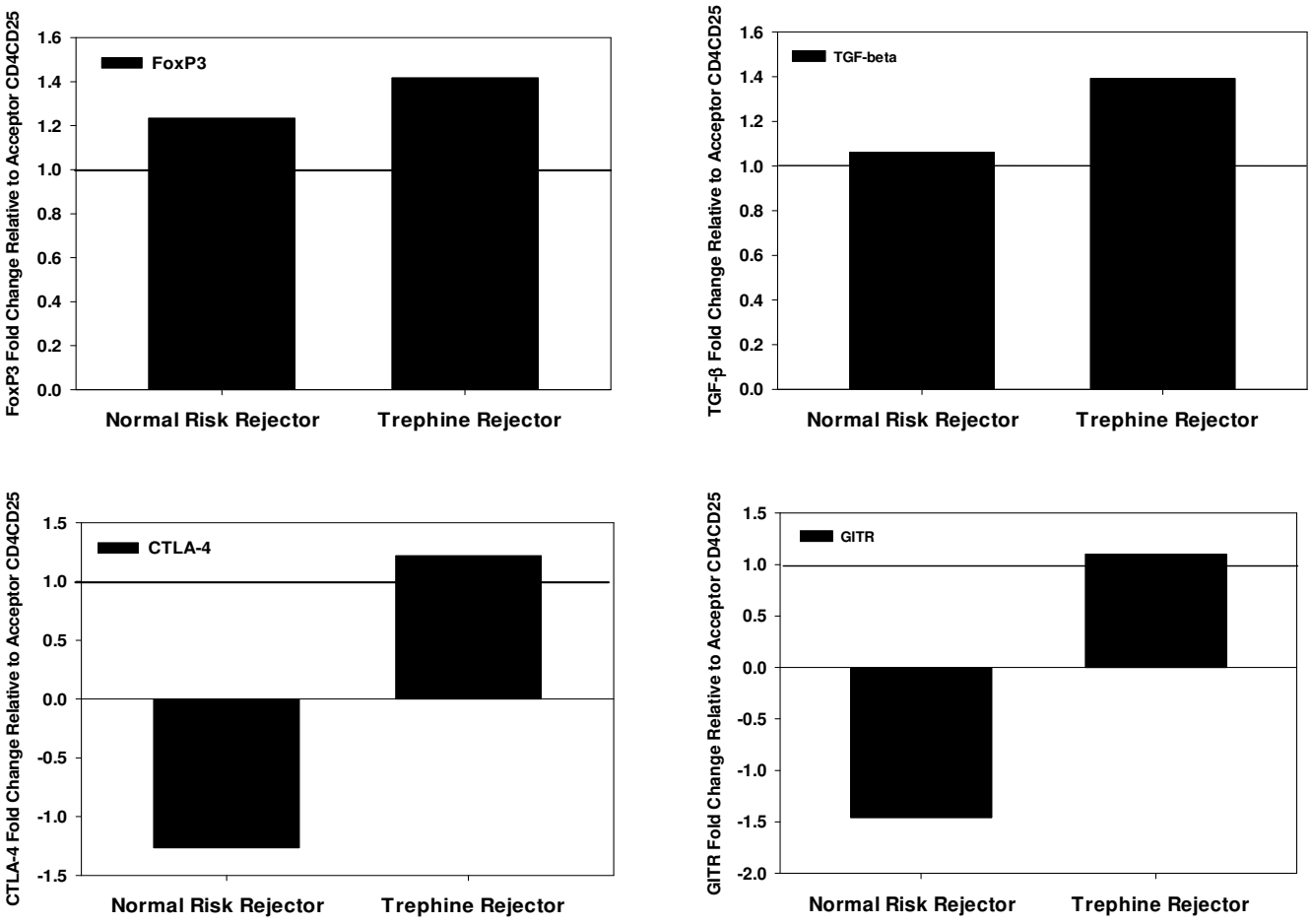


Figure 27. Disruption of the corneal nerves does not affect the transcription of suppressive molecules on $CD4^+CD25^+$ T cells. The mRNA expression of A.) FoxP3, B.) TGF-β1, C.) CTLA-4, and D.) GITR was assessed on $CD4^+CD25^+$ T cells 72 hours following anti-CD3ε and anti-CD28 stimulation. This experiment was performed 4 separate times with similar results. $P > 0.05$ for all suppressive molecule expression.

Chapter Four

Discussion

Clinical Relevance of Current Study

Corneal transplantation is one of the most successful forms of solid transplantation in humans. The 90% two year survival rate of corneal transplants is exceedingly high considering it is conducted under low stringent conditions. Routinely, corneal transplants are performed without histocompatibility matching between the donor and the recipient. Additionally, there is no use of immunosuppressive therapy; rather patients are treated with topical corticosteroids for the first 2 years. The high success of corneal allografts under low stringent conditions is attributed to the immune privilege of the eye. The immune privilege of the eye is comprised of three main mechanisms: 1.) preventing the induction of the alloimmune response; 2.) deviating the immune response towards tolerance and 3.) preventing effector cells from infiltrating into the local ocular environment to mediate the destruction of the allografted cornea [32]. However, the fidelity of immune privilege is not absolute, as approximately 10-20% of the corneal transplants will still fail due to immune-mediated rejection. Moreover, patients with pre-existing conditions, such as prevascularization of the graft bed or ongoing allergic diseases have a higher incidence of graft rejection [227].

When a corneal allograft rejects, a patient may elect to have the failed tissue replaced with a new healthy cornea, especially if the affected eye is the patient's only eye useful for vision. However, electing to receive a second corneal transplant comes with increased risk. Undoubtedly, patients that require multiple corneal transplants are at the highest risk of rejection compared to other patients. When a corneal allograft is placed onto the same eye, the long term

risk of rejecting a corneal allograft increases with each successive corneal transplant (second – 55%; third – 75%; fourth – 100%) [176, 193, 228].

Since there is no evidence that verifies histocompatibility matching the donor tissue to the host improves graft survival, corneal tissues are selected solely on the basis of the quality of the tissue, rather the MHC genotype of the cornea donor. However, as there are no records of the histocompatibility genotype of most corneal transplants performed in the U.S., it is impossible to comment on whether first corneal allografts and second corneal allografts share any histocompatibility markers. Thus, it is unknown if patients requiring multiple corneal transplants have a higher incidence of graft rejection due to prior sensitization and the presence of a memory immune response that would recognize the second corneal allograft. The present study investigated if prior sensitization was the mechanism accounting for the increased incidence of rejection that occurs in hosts that receive multiple corneal allografts. This study demonstrates that hosts requiring multiple corneal transplants, either on the same eye or on the contralateral eye, are at increased risk of graft rejection of the second graft and the first graft. The increased incidence of rejection was neither due to shared alloantigens nor due an immune response towards eye-specific antigens. We hypothesized that recipients of multiple corneal transplants have a perturbation of immune privilege due to the corneal transplantation procedure itself. This study addressed how the corneal transplantation procedure abrogates immune privilege. We delineated how the two major manipulations performed during the corneal transplantation process, the incision through the host's corneal tissue and the suturing of the donor tissue, abrogate one or multiple mechanisms that support ocular immune privilege. This investigation will provide further insight into the development of future therapies that will extend the survival of corneal allografts in recipients requiring multiple corneal transplants.

PRIOR SENSITIZATION IS NOT REQUIRED FOR INCREASED GRAFT REJEJCTION IN HOSTS RECEIVING TWO CORNEAL GRAFTS

Shared alloantigens and eye-specific antigens do not have a role in promoting graft rejection

Testing for tissue histocompatibility and serum antibody alloreactivity has clearly enhanced the prognosis of kidney, heart and liver transplantation. Patients that have a low antibody alloreactivity response are less likely to recognize the allografted tissue and have a better chance of accepting the transplanted tissue [229]. These steps prior to transplantation predict the patient's propensity to generate donor-specific HLA antibodies and develop a hyperacute immune response towards the allograft. However, histocompatibility matching at the MHC or mH gene loci has not shown any beneficial effect in normal corneal allograft recipients [230]. Thus, under normal conditions, a first time corneal allograft recipient will not be matched with the donor tissue, but rather the patient will receive a corneal graft selected for its suitability for transplantation based primarily on the quality and integrity and cell density of the endothelial layer [231, 232].

Of the 40,000 corneal transplants performed each year, 10% of these will undergo graft rejection and will require a subsequent replacement corneal allograft. During the rejection process, alloantigens expressed on the transplant sensitize the recipient and stimulate the generation of alloantigen-specific $CD4^+$ T cells that are capable of mediating a DTH response [104]. Upon receiving the second corneal allograft, the memory $CD4^+$ T cells encounter APCs that present the corneal alloantigens to the memory CD4+ T cells. Alternatively, memory $CD4^+$ T cells localized within the host's corneal tissue will directly recognize the alloantigen presented by the second graft. The reactivation of the memory $CD4^+$ T cells results in a more efficient and more pronounced inflammatory response that hastily mediates the rejection of the second graft.

It has been speculated that prior sensitization to shared alloantigens between the first and second corneal allografts is the mechanism mediating the increased incidence of rejection in the second graft [196]. We addressed this issue by developing a murine model of repeat corneal transplantation, where the initial A/J or C3H donor does not share any histocompatibility molecules, at either the MHC or mH loci, with the subsequent B6 corneal allograft donor. Recipients that rejected an initial A/J or C3H corneal allograft had an increased incidence of rejection of subsequent B6 corneal allografts transplanted onto the rejected eye or the contralateral eye. This demonstrates that an alloantigen-driven memory response is not required for the increased incidence of rejection of a subsequent corneal allograft. These result correlate with the study conducted in by Nicholls et. al. to determine if corneal allografts disparate at the MHC loci still instigate a high incidence of graft rejection [233]. They noted that PVG rats that rejected AO corneal transplants experienced a high incidence of rejection of subsequent LEW corneal allografts transplanted onto the initial eye or the contralateral eye [233]. Together, these data refute the hypothesis that prior sensitization from the initial corneal allograft is the mechanism required to cause the heightened incidence of graft rejection of the subsequent corneal allografts in repeat corneal allograft recipients.

It is now known that after T cell clonal expansion and contraction, a population of memory T cells with a heterogeneous TCR repertoire remains [234]. Current research suggests that memory immune responses towards a specific antigen from a prior infection can result in cross-reactivity towards an unrelated epitope and promote autoimmunity and allograft rejection [234-238]. Nicholls et. al. noted that rejectors of an AO and LEW corneal allograft had the same tempo of rejection of a subsequent LEW corneal allograft [198] This suggested that an AO graft was as efficient as a LEW graft in its ability to sensitize recipients towards a subsequent LEW

allograft and to expedite graft rejection. Moreover, lymphocytes from recipients that rejected LEW corneal allografts had positive T cell proliferative responses towards the previously unseen AO antigens [233]. However, this study did not address if the memory $CD4^+$ T cells from the initial AO corneal transplant recognized mH alloantigens presented on the subsequent LEW corneal allograft or if this response was due to cross-reactivity. We addressed the hypothesis that the elicitation of a cross-reactivity response mediated high incidence of rejection by eliminating the induction of the initial immune response that generates alloantigen memory $CD4^+$ T cells. The transplantation of a syngeneic BALB/c corneal graft, which does not express any alloantigens, enhanced the rejection of a B6 corneal allograft placed onto either eye. These results suggest that the activity of alloantigen-induced memory $CD4^+$ T cells that cross-react to third-party alloantigens is not responsible for the heightened incidence of rejection of the subsequent corneal allografts. Since there were no shared alloantigens expressed on the initial and second corneal transplants we wanted to elucidate if the increased incidence of rejection was an immune-mediated response towards alloantigens or towards autoantigens expressed exclusively within the eye.

Medawar attributed the immune privilege of the eye to the lack of lymphatic drainage, which effectively sequesters the ocular tissue and the antigens it expresses from the peripheral immune system [28]. Ocular antigens normally sequestered within the eye reside behind the ocular-blood barrier and would escape exposure to the peripheral immune system. Thus, T cells with a TCR repertoire that reacts towards tissue-specific antigens expressed specifically within the eye would not be eliminated in the thymus during the central tolerance process and would remain in circulation in normal hosts [239]. Moreover, the absence of blood vessels within the cornea limits the access of the ocular antigen-reactive T cells into the ocular tissue thereby

limiting the circulating T cells access to their cognate antigens expressed within the eye. The sequestration of ocular antigens may inhibit the induction of peripheral tolerance and predispose the induction of inflammatory reactions towards tissue-specific antigens expressed in the eye [216]. Thus, it was believed the immune system was 'ignorant' of ocular antigens due the lack of blood and lymphatic vessels.

However, it is believed the trauma induced by a penetrating ocular injury or surgery to one eye abolishes the ocular-blood barrier through the infiltration of blood and lymphatic vessels, allowing the release and exposure of eye-specific antigens into the peripheral immune system [240]. Recruited APCs would be free to phagocytize the eye-specific antigens and leave the ocular environment and migrate to the spleen and draining lymph nodes. In these two lymphoid organs, the APCs could present the eye-specific antigens to $CD4^+$ T cells, and selectively activate autoreactive $CD4^+$ T cells. NO and ROS upregulate receptors on vascular endothelial cells, thus enabling activated $CD4^+$ T cells to migrate back through the blood vessels into the injured eye or the contralateral eye [240]. This autoinflammatory effect results in the infiltration of macrophages and activated $CD4^+$ T cells within the choroid and the formation a granuloma in the uninjured eye, a condition referred to as sympathetic ophthalmia [214, 241, 242]. Since we noted that even a syngeneic graft increased the incidence of rejection of a second graft in either eye, it remained to be determined if the corneal transplantation procedure enabled the release of eye-specific antigens to the peripheral immune system and elicited an auto-reactive immune response towards subsequent grafts. However, we did not observe an enhanced immune reaction towards a subsequent syngeneic graft transplanted onto either the previously grafted eye or the contralateral eye. These results are in agreement with Maumenee's observations that corneal autografts did not elicit an immune response and undergo rejection in the second eye

[243]. He concluded from these results that the rejection of corneal allografts was not due to the 'organ-specific type of sensitivity to corneal proteins'[243]. In fact, when the risk of corneal allograft rejection is high and a portion of the cornea is clear, an autokeratoplasty of the patient's own tissue can be re-grafted onto the same eye or transplanted onto the other eye. In either case, the procedure is conducted without the complications associated with allograft transplantation and results in restoration of vision [244-246]. This suggests that the corneal transplantation procedure does not elicit an autoimmune hypersensitivity response towards eye-specific antigens that leads to the rejection of subsequent corneal grafts.

The initial aim of this project was to determine if prior sensitization from an initial corneal allograft induced a memory response that would consistently be recalled after each subsequent corneal allograft and lead to the increasing incidence of graft rejection. However, these results demonstrate that prior sensitization toward alloantigens is not required for the high rejection incidence of subsequent corneal allografts. Moreover, we observed that the corneal transplantation procedure did not incite an auto-reactive immune response toward eye-specific antigens and did not lead to an inflammatory response towards a syngeneic corneal graft. Our observations suggest that the corneal transplantation procedure itself leads to a loss of immune privilege, not only in the transplanted eye, but also in the contralateral eye. The second goal of this study was to determine how the corneal transplantation procedure abrogates immune privilege in both eyes, and the mechanism that leads to the loss of tolerance.

SURGICAL MANIPULATIONS LEAD TO THE LOSS OF IMMUNE PRIVILEGE

Since a syngeneic graft promotes corneal allograft rejection, but not failure of a syngeneic corneal graft, we hypothesized that one or many manipulations performed during the

corneal transplantation procedure led to the loss of immune privilege. Thus, we aimed to determine which step of the corneal transplantation procedure abrogates immune privilege, not only in the grafted eye, but also in the contralateral eye. The steps of the corneal transplantation procedure can be broken down to two main manipulations; 1.) the excision of the recipient's affected cornea and 2.) the securement of the corneal transplant. We wanted to establish how these specific manipulations performed during the corneal transplantation procedure would affect immune privilege in the local grafted eye and the sympathizing contralateral eye.

Suture induced angiogenesis abrogates local immune privilege

The securement of the corneal graft with sutures can have a detrimental effect by acting as an irritant, which can promote edema and vessel infiltration into the corneal tissue and ultimately to the rejection of a corneal graft [247, 248]. We assessed the isolated effect sutures from a previous corneal graft had on immune privilege in the manipulated eye and in the contralateral eye. We observed that the placement of sutures through the stroma of the cornea instigated the infiltration of blood vessels into the sutured cornea, but not the opposite cornea. The placement of the corneal allograft into the highly vascularized corneal graft bed enhanced graft rejection. Our results coincide with previous results, demonstrating the placement of sutures instigates the infiltration of blood vessels into the corneal tissue and results in high incidence of allograft rejection of a corneal transplanted into the same eye [188, 189, 249]. One of the predominant features of the immune privilege of the eye is the lack of infiltrating blood and lymph vessels within the cornea. However, enhanced vascularization of the cornea, either from a preoperative condition or secondarily from the effects of a prior ocular surgery, can break immune privilege and is a predominant risk factor for graft rejection [250]. Previous reports suggest that the sole presence of blood vessels into the cornea enables donor and host APCs to

migrate into the regional lymph nodes and allowing the infiltration of $CD4^+$ T cells into the corneal allograft [39]. However, recent studies showed that the stimuli that instigated the infiltration of blood vessels also induced a concomitant infiltration of lymphatic vessels [251-254]. VEGF-A within the eye mediates the recruitment of macrophages, which release the lymphangiogenic factors VEGF-C and VEGF-D, and induces not only the infiltration of blood vessels but also lymph vessels into the corneal tissue [255]. VEGFR2, endogenously produced within the cornea, maintains the avascularity of the cornea. By selectively ablating the expression of VEGFR2 within the cornea, the tissue becomes saturated with lymph vessels but not with blood vessels [181]. The importance of lymphangiogenesis over hemangiogenesis as a predominant risk factor for graft rejection was demonstrated when the administration of soluble VEGFR2, which inhibited lymph vessel formation but retained the suture induced infiltration of blood vessels, enhanced graft survival [181]. These results are in agreement with a study that showed the administration of an $\alpha5\beta1$ antagonist, which effectively blocked lymphangiogenesis and not hemangiogenesis, reduced graft in recipients that received corneal sutures [188]. These results emphasize that the high incidence of graft rejection due to intrastromal suturing is due to the influx of lymph vessels into the corneal tissue.

Lymphangiogenesis of the corneal tissue provides a conduit for APCs to migrate to the regional lymph nodes and induce an immune response through the generation of alloreactive $CD4^+$ T cells. However, these allospecific $CD4^+$ T cells must also be recruited back to the corneal tissue to carry out the destruction of the corneal allograft. Amescua et. al. noted that the placement of sutures not only promoted corneal vascularization, but also stimulated the production of the T cell chemokines CXCL1/KC, CXCL9/Mig, and CXCL10IP10 [189]. By neutralizing CXCL1/KC, corneal allografts transplanted onto a sutured graft bed had a survival

status comparable to a corneal allografts transplanted onto an unmanipulated eye. Thus, the placement of sutures abolishes immune privilege by allowing the infiltration of blood and lymph vessels into the corneal graft bed, and allows the influx of inflammatory APCs and alloreactive T cells into the corneal allograft.

The perturbation of immune privilege by corneal sutures was not extended to the contralateral eye. The contralateral eye retained its avascularity and was devoid of blood and lymphatic vessels in the corneal tissue. Although vascularization of the eye is a high risk factor for graft rejection, the presence of vessels only affects local immune privilege. That is, suturing of the graft bed abolishes local immune privilege but not immune privilege of the contralateral eye. The loss of immune privilege in the contralateral eye cannot be explained by the presence of new lymphatic vessels that enhance sensitization of the host to the corneal allograft.

Circumferential incisions abrogate local and sympathetic immune privilege

The other manipulation performed during the corneal transplantation procedure is the excision of the host's diseased cornea. This process, involving the use of a trephine to create a circumferential incision through the epithelium and stroma, breaks many of the microanatomical structures of the cornea and disrupts the integrity of the epithelial cell layer, decreases corneal sensation and disrupts tear secretion [256, 257]. The non-penetrating circumferential incision through the epithelium and into the anterior aspect of the stroma resulted in the local ablation of the subbasal nerve plexus innervating the central cornea. In laser-assisted in situ keratomileusis (LASIK) surgery, a circumferential incision is performed and also results in the dramatic loss of central corneal nerves. The nerve fiber density is dramatically decreased for up to 5 or more years post LASIK surgery, and may never return to pre-operative levels [258]. Moreover, the

effects of LASIK can be detrimental, resulting in neurotrophic keratopathy, tear instability, tear deficiency and neuropathic pain, and culminate in dry eye disease [259-261]. Streilein et. al. also demonstrated that both a circumferential and X-shaped incision of the central cornea reduced the levels of immunosuppressive factors that were capable of suppressing T cells [203]. The destruction of corneal nerves with a circumferential incision, but not an X-shaped incision, resulted in a reduced capacity to induce ACAID [203]. The damage incurred by a circumferential incision of the cornea stimulates the wound healing response to remove necrotic cells and allow the cornea to regenerate its normal architecture by reestablishing the epithelial layer and depositing new stromal collagen. The early onset of corneal wound healing responses following LASIK are characterized by macrophage infiltrates that engulf cellular debris and the upregulation of growth factors and cytokines such as epidermal growth factor (EGF), keratinocyte growth factor (KGF), TGF-β, IL-1, IL-6 and TNF-α [262]. These growth factors and cytokines modulate the wound healing response by promoting epithelial cell proliferation and motility [263]. However, the cytokines TNF-α IL-1 and IL-6 can have deleterious effects by promoting inflammatory responses in the cornea. IL-1 stimulates the chemotactic factor IL-8 production by corneal epithelial and stromal cells and induces LC migration [264, 265]. The wound healing response from the prior circumferential incision predisposes the increased phagocytic activity of macrophages and motility of LCs to enter into the allograft and uptake of alloantigen [266]. The preferential expression of IL-1, IL-6, and TNF-α within the wounded corneal tissue counteracts the immunomodulatory effects of TGF-β and prejudices the APC population towards a pro-inflammatory population capable of inducing alloimmunization and ultimately, allograft rejection [266-268]. Thus, the circumferential incision's wound healing response might break local immune privilege through the upregulation of proinflammatory

cytokines and the enhanced APC function of residential macrophages and LCs. It remains to be determined if the wound healing response induced by the X-shaped incision, without the destruction of the subbasal corneal nerves, also mediates the loss of local immune privilege. Our results further exemplify that a circumferential incision through the corneal epithelium and stroma disables local immune privilege and results in the loss of AC immune privilege.

Our results demonstrate that circumferential incisions also abolish immune privilege in the contralateral eye. However, the inflammatory effects produced by the wound healing response of one cornea did not extend into the contralateral eye, as an X-shaped incision in one eye did not promote a significant increase in graft rejection in either eye. The defining feature that distinguishes the circumferential incision from the X-shaped incision was the ablation of the corneal nerve plexus. Streilein et. al. observed that the circumferential incision, but not an X-shaped incision, ablates the innervation process into the central cornea and disables the establishment of ACAID in the same eye [203]. Our study verifies that the ablation of corneal subbasal nerve plexus from a circumferential incision, but not an X-shaped incision, results in the loss of immune privilege, not only in the incised eye but also into the contralateral eye.

Ablation of the corneal nerves alters immunoregulatory neuropeptide expression

The next aim of this study was to establish how the destruction of the corneal nerves disrupts immune privilege and results in enhanced corneal allograft rejection. The ocular nerves are important for maintaining a healthy cornea by stimulating blink reflex responses and regulating epithelial cell renewal. However, herpetic viral infections, trigeminal nerve damage, and corneal surgeries disrupt corneal innervation [269-271]. Without the neuropeptides supplied by the nerves, the patient's cornea develops ulcerations and perforations resulting in the

desiccation of the corneal surface [7]. Moreover, the neurotransmitters of the cornea maintain the homeostatic environment by exerting their immunomodulatory effects to regulate the immune privilege of the eye [224, 272]. This is not the first study to demonstrate that unilateral neuropathic inflammatory response due to surgical injury or infection leads to a bilateral response, resulting in an alteration of the homeostatic environment in the contralateral eye [273, 274]. Patients undergoing vitrectomy have significantly higher AqH flare levels in the contralateral eye two weeks after the procedure [275]. Patients that underwent phacoemulsification or trabeculectomy had increased flare and cellular infiltrate in the unmanipulated eye for seven days. Twenty-eight days after the procedure, the AqH returned to pre-operative baseline levels [276-279]. In these cases, no pathological condition leading to the loss of vision was detected, suggesting that a surgical manipulation to one eye has a subclinical effect in the other eye.

Our studies demonstrate that a circumferential incision to one eye alters the microenvironment of the contralateral eye by disrupting the level of the immunomodulatory neuropeptides. We noted that the upregulation of the pro-inflammatory neuropeptide SP and the downregulation of the tolerance-inducing neuropeptide α-MSH could direct the immune response away from tolerance and provoke inflammation.

Reconstitution of α-MSH does not enhance graft survival in mice subjected to circumferential corneal incisions

The neuropeptide α-MSH is contained within the AqH and suppresses ROS production by macrophages and IFN-γ production by T cells [47]. One group suggested the local administration of α-MSH suppresses the expression of IL-2 and IFN-γ, reduces the recruitment

of leukocytes to the corneal allograft, and reduces the DTH response against the allograft [280]. These investigators found that providing exogenous α-MSH locally via subconjunctival injections augmented immune privilege by inhibiting the alloimmune response and as a result, enhanced graft rejection in normal corneal allograft recipients [280]. Furthermore, recent evidence suggests that α-MSH can induce tolerance by converting $CD4^+$ T effector cells to $CD4^+FoxP3^+CTLA\text{-}4^{Hi}$ Tregs [281]. Since circumferential incisions downregulated α-MSH in the contralateral eye and prevented the generation of functional Tregs we hypothesized that local reconstitution of α-MSH in the contralateral eye would restore immune privilege and promote graft survival. Initially, we injected α-MSH subconjunctivally as a means of restoring immune privilege, but the injection caused extensive inflammation. Accordingly, α-MSH was provided systemically by i.p. injections to circumvent trauma induced the inflammatory reactions. We followed the same regimen that has been shown to alleviate EAU [225]. Intraperitoneal injections of several different amounts of α-MSH did not enhance graft survival in either normal risk hosts or in hosts treated with circumferential corneal incisions. It remains to be determined if the downregulation of α-MSH that occurs following circular corneal incisions has any physiological impact on the enhanced incidence of graft rejection in circumferentially incised corneal allograft recipients.

Injection of SP recapitulates the exacerbated corneal allograft rejection that occurs in hosts subjected to circumferential corneal incisions.

Since the creation of a circumferential incision to one eye upregulated the level of SP in the contralateral cornea, our investigation focused on the possible impact of SP on the loss of immune privilege. Stein-Streilein et. al. showed that a laser burn in the retina of one eye is

perceived in the contralateral retina through the upregulation of the SP receptor, NK-1R, and results in the disruption of ACAID in the unburned eye [69]. By inhibiting SP activity through the administration of Spantide I or the use of $SP^{-/-}$ mice, the abolition of immune privilege was reversed and ACAID was restored [69]. Our study re-affirmed the role of SP as an inflammatory neuropeptide that abolishes ocular immune privilege and exacerbates corneal allograft rejection. Administration of SP, both through the local ocular environment or systemically via intravenous injection, abolished immune privilege and resulted in the exacerbation of corneal allograft rejection. Moreover the administration of the NK-1R antagonist, Spantide II, in recipients that received circumferential corneal incisions restored immune privilege and confirmed the physiological relevance of SP in the increased severity of corneal allograft rejection in hosts receiving second corneal grafts.

Our studies aimed to determine the onset and the duration of the loss of immune privilege after a circumferential corneal incision. It is important to determine for patients who require bilateral corneal transplantation if prolonging the time after the initial corneal allograft would be beneficial for the survival of both the subsequent and initial corneal allograft. Obzek et. al. observed that a corneal allograft in the second eye did not impose a greater rejection risk of the initial corneal allograft if the second transplantation was delayed for at least 18 months [282]. Our results suggest the release of SP as a result of the destruction of the corneal nerves of one eye leads to an acute and long-term loss of immune privilege for at least 100 days. The proposed mechanism as to how the release of SP in response to circumferential corneal incisions abrogates short-term and long-term corneal allograft tolerance is discussed below.

Locally produced SP mediates an acute loss of immune privilege

Our initial studies suggest that the ablation of corneal nerves sends a neurological signal to the contralateral eye and maintains the upregulation of SP for at least 14 days. This study established that the transient exposure of the ocular environment to SP, through a single bolus subconjunctival injection, can temporarily inhibit the induction of immune privilege. SP within the ocular environment could have enhanced the acute form of rejection by stimulating pro-inflammatory cell infiltration, and lymphocyte proliferation and resistance to apoptosis. SP can promote local vascular permeability and angiogenesis [283, 284], yet we did not consistently note the infiltration of blood vessels within the contralateral eye prior to transplantation. However, the sustained upregulation of SP for at least 14 days within the contralateral eye does not rule out the possible role of SP in promoting a more vigorous infiltration of blood vessels into the corneal allograft after transplantation. Since SP can break the ocular-blood barrier by increasing vascular permeability of the blood vessels and the expression of chemokines such as MIP-1 and MCP-1, which are associated with corneal allograft rejection [285-287], the graft would become accessible to infiltrating macrophages and $CD4^+$ T effector cells. Additionally, SP may promote the proliferation and survival of infiltrating inflammatory cells. One study indicated that SP signaling through NK-1R on glioblastoma cells increases the phosphorylation and the activity of Akt via phosphatidyl-3-kinase, which suppresses apoptosis [288]. Corneal tissues express pro-apoptotic molecules such as FasL, PD-L1, and TRAIL to protect them from inflammatory macrophages and $CD4^+$ cells. It is through the induction of cell death that immune privilege is established. Ferguson et. al found that animals raised in the dark had an upregulation of SP, had very few apoptotic cells following AC injection of antigen and could not support ACAID [70]. Moreover, they noted that T cells pretreated with SP prior to stimulation with

phorbol ester and ionomycin were resistant to apoptosis [289]. Zhou et al. observed SP anti-apoptotic effects in the cornea, by noting that blockade of SP promoted macrophage apoptosis in *Pseudomonas aerouginosa* infected corneas [290]. Therefore, circumferential incisions upregulate SP, which upon interaction with the infiltrating $CD4^+$ T cells and macrophages, promotes their survival by inhibiting the apoptotic death pathway. This renders the infiltrating inflammatory cells impervious to the pro-apoptotic molecules FasL, TRAIL, and PD-L1, which are expressed on the corneal endothelium and enables the immune cells to infiltrate into the graft and mediate the destruction of the corneal allograft. However, the localized effect of SP, as exemplified by of a single bolus injection, does not explain why a circumferential incision 60 days prior to transplantation still results in the high incidence of corneal allograft rejection.

Systemic SP mediates a long-term loss of immune privilege

We observed that severing the corneal nerves of one eye mediated a loss of immune privilege in the other eye for up to 100 days. This observation coincides with Stein-Streilein's study that showed 68 days after a retinal laser burn to one eye, the contralateral eye could not support the induction of ACAID. The loss of immune privilege coincided with the upregulation of NK-1R in the contralateral retina [69]. Delivering SP to the ocular environment mediates only a short-term loss of immune privilege. By contrast, when delivered systemically, SP mediates an immediate and long-lasting loss of immune privilege. Hong et. al. showed that alkali burns to the cornea of one eye result in an upregulation of SP serum levels [291]. The increased level of SP in the periphery acts as a chemoattractant, and promotes stem cell progenitor cells to migrate from the bone marrow (BM) and travel to the injured cornea [291]. SP can have long-term immune effects by modulating BM-derived hematopoietic cell populations. It is through the

BM-derived hematopoietic cell lineage that the immune and blood cells are generated. Tachykinin positive nerve fibers found within the bone marrow, release SP [292-294] and are important for the hematopoiesis of BM-derived stem cells and hematopoietic progenitor cells [295, 296]. SP promotes the expression of IL-1, IL-13, GM-CSF, and SCF, which regulate the commitment of hematopoietic progenitor cells into the myeloid lineage [297-299], stimulate progenitor BM cell proliferation [300], and stimulate the migration of progenitor cells from the BM through the upregulation of CXCR4 [301]. Since the bone marrow is the source of immune cells and is required for a functional immune system, the influence of SP may bias a pro-inflammatory response, rather than a tolerogenic response [302, 303]. Since SP promotes the expression of its receptor NK-1R [304], it stands to reason that the inflammatory cell population produced in response to SP may become more sensitive to subsequent encounters with SP, and thus be prone to follow an inflammatory pathway and not a tolerogenic response. As both T cells and APCs express NK-1R [60, 305, 306], SP within the local environment of the grafted eye can deviate these cells from a tolerogenic phenotype to an inflammatory phenotype and stimulate graft rejection. The elevated level of SP in the periphery, both by injection of SP i.v. or the destruction of the corneal nerves in one eye, may enhance the hematopoietic process and increase the population of circulating inflammatory cells that express increased sensitivity to the secondary release of SP from a second corneal transplant. This suggests that injury to the corneal surface may also promote the systemic release of SP and fundamentally alter the activity of BM-derived cells. This hypothesis is consistent with our findings, which indicate that either circumferential corneal incisions or intravenous injection of SP results in a long-lived enhancement of corneal allograft rejection that persists for at least 100 days.

SP breaks immune privilege through the abrogation of Treg suppressive function

It was important to elucidate how SP abolishes immune privilege and promotes corneal allograft rejection. $CD4^+$ T cell play a crucial role in corneal allograft rejection through the recruitment of macrophages, the production of IFN-γ and TNF-α and ultimately in the mediation of DTH responses against corneal alloantigens [104, 123, 307]. We did not know if the loss of immune privilege in mice subjected to circular corneal incisions was due to the direct interaction of SP on $CD4^+$ T cells which express NK-1R [306]. Multiple studies have demonstrated that nanomolar concentrations of SP stimulate $CD4^+$ T cell proliferation [308-310]. Additionally, $NK\text{-}1R^-CD4^+$ T cells have consistently lower proliferative responses compared to their $NK\text{-}1R^+CD4^+$ WT counterparts [310-313]. In the presence of SP, $CD4^+$ T cells have enhanced production of IL-2 and display elevated lymphocyte proliferation [313, 314]. This would suggest that SP might amplify the inflammatory response by simply enhancing the activation and proliferation of $CD4^+$ T effector cells. However, we observed that $CD4^+$ T cells stimulated with SP did not have a significantly enhanced DTH response.

Our group, as well as others, has shown that the induction of tolerance through the generation of $CD4^+CD25^+$ Tregs is vital for the survival of corneal allografts [141, 143, 158, 161]. For example, disabling Tregs through the administration of anti-CD25 antibody abolished tolerance and provoked corneal allograft rejection [141]. Moreover, the adoptive transfer of $CD4^+CD25^+$ Tregs into corneal allograft recipients results in enhanced graft survival [161]. Further examination showed that corneal allograft rejectors have the same frequency of $CD4^+CD25^+$ $FoxP3^+$ T cells as those that accepted their corneal allografts. The differential factor between the corneal allograft recipient $CD4^+CD25^+$ T cell populations was reflected in the

heightened level of FoxP3 expression in acceptors compared to rejectors [161]. The $CD4^+CD25^+$ Tregs require direct contact with $CD4^+$ T effector cells to induce tolerance, as blocking their function via neutralization of CTLA-4, m-TGF-β, and GITR diminished their suppressive activity [158].

Allergic conjunctivitis abolishes immune privilege of corneal allografts and results in a steep increase in graft rejection [151, 315]. The IL-4 produced by mice with allergic conjunctivitis renders alloantigen-specific T effector cells resistant to Treg suppressive function [149]. Early studies have shown that SP can induce T cells to produce Th2-associated cytokines such as IL-4 and IL-10 [316]. We tested the hypothesis that SP acts directly on $CD4^+$ T cell populations and renders the alloantigen-specific $CD4^+$ T cell population resistant to Treg-mediated suppression. Our results showed that $CD4^+$ effector cells from mice treated with circumferential corneal incisions were still susceptible to Treg-mediated suppression. This finding suggests that SP does not promote graft rejection by rendering the alloantigen-specific T effector cells resistant to Treg-mediated suppression.

We observed that the presence of elevated levels of SP prevent corneal allograft recipients from generating functional $CD4^+CD25^+$ Tregs. Our study demonstrates that the ablation of the corneal nerves and the resulting release of SP disrupt the induction of functional $CD4^+CD25^+$ Tregs. The induction of corneal transplant tolerance requires the participation of contact-dependent $CD4^+CD25^+$ Tregs [158]. Accordingly, we assessed the contact-dependent suppressive molecules that were the most potent for Treg-mediated suppression in the corneal allograft setting; CTLA-4, GITR, m-TGF-β, and FoxP3. However, the expression of these molecules was the same in normal risk acceptors, normal risk rejectors, and high risk rejectors

that were treated with circumferential corneal incisions. This study generated different results than those conducted by Cunnusamy et. al. where the inability to generate functional Tregs was correlated with reduced expression of CTLA-4, TGF-β, and GITR [156]. That study found that IL-17A promoted the generation of $CD4^+CD25^+$ Tregs, and the absence of IL-17A resulted in a reduced expression of membrane bound suppressive molecules. It is unknown if the inability to generate $CD4^+CD25^+$ Tregs in our study was due to the lack of IL-17A in circumferentially incised recipients. However, SP can promote $CD4^+$ T cells to produce IL-17 [317], the cytokine implicated in the generation of Tregs for corneal transplantation acceptance [158]. Thus, the weight of evidence suggests that the upregulation of SP in mice treated with circumferential corneal incisions inhibits the generation of Tregs through a mechanism independent of IL-17A.

This is not the first study to demonstrate functionally impaired $CD4^+FoxP3^+$ T cells. $CD39^+$ Tregs have been shown to be stable in inflammatory reactions [318], and the reduction of CD39 expression on $FoxP3^+$ Tregs has been reported in patients with hepatitis, multiple sclerosis, and systemic lupus erythematosus [319-321] and correlates with reduced suppressive function. Moreover, it should be noted that the function of TGF-β is dependent on its conversion from its latent form into the active form, not solely on the level of TGF-β protein expression. TGF-β is synthesized in the inactive form and is associated with latent TGF-β-associated peptide (LAP). The presence of thrombospondin, integrins β1, β6, and β8, ROSs and estrogen can activate the latent form of TGF-β by releasing LAP and exposing the TGF-βR binding site [322, 323]. Thus, one explanation to account for the dysfuction of the $CD4^+CD25^+$ T cell population in mice subjected to circular corneal incisions is the failure to convert TGF-β from its latent to an active state.

As professional APCs, dendritic cells (DCs) play a crucial role in the induction of T cell immunity and tolerance. DCs also express the SP receptor NK-1R, [324] which signals through NFκB and promotes the expression of inflammatory cytokines, such as IL-1β, IL-6, IL-12, and TNF-α [325]. SP promotes APCs to upregulate the expression of MHC class II; the co-stimulatory molecules CD80, CD86, and CD40; and the cell adhesion molecules CCR7, CD11b, CD18 and CD54 [311, 326-328]. This suggests that DCs exposed to SP become better equipped to migrate to the draining lymph node, interact with and stimulate T cells, and induce a robust Th1 pro-inflammatory immune response capable of mediating corneal allograft rejection. Thus, the inability to properly convert $CD4^+CD25^+$ T cells to functionally suppressive Tregs may be due to flawed interactions between T cells with DCs.

Abrogation of Established Immune Privilege

Influence of a second corneal allograft on the function of existing Tregs

A prospective study on bilateral corneal allograft recipients by Williams et. al. examined if a rejection episode in either grafted eye would affect the risk of rejection in the other grafted eye [176]. They found that a graft in the second eye was 3 times more likely to be rejected if a rejection episode occurred in the first eye, even though the donors of the two grafts were unrelated and presumably did not share any histocompatibility antigens. Moreover, the first allograft was 2 times more likely to be rejected if a rejection episode occurred in the second eye [329]. These findings are consistent with our findings, which demonstrate that initial clear corneal allograft can undergo the rejection after a second corneal graft is transplanted onto the opposite eye. Our results further show that the rejection of the initial corneal allograft is not due to a bystander immune response against the second corneal allograft. Rather, the destruction of

the corneal nerves in the second eye renders the initial corneal allograft vulnerable to immune rejection.

The findings of the current study show that the ablation of the nerves in one eye promotes the hasty rejection of an otherwise established clear corneal allograft in the contralateral eye. A single bolus injection of SP into the conjunctiva of an eye bearing a clear corneal allograft breaks immune privilege and results in graft rejection. Moreover, one pg. of SP abrogates the suppressive function of otherwise functional $CD4^+CD25^+$ Tregs. Ours is the first study to show that SP promotes immune-mediated inflammation by inhibiting Treg function. The loss of Treg suppressive function can be attributed to the plasticity of T cells and their ability to differentiate to an alternative phenotype with different functions [330]. A recent study suggests SP can act on memory $CD4^+CD40RO^+IL\text{-}23R^-$ T cells and promote their differentiation to $ROR\gamma\tau^+IL\text{-}17^+$ Th17 cells [317]. Thus, it could be hypothesized that the loss of Treg suppressive function is due to the plasticity of the Tregs and upon interaction with SP they differentiate into T effector cells. Our data suggests that SP can abrogate established immune privilege by inhibiting the function of $CD4^+CD25^+$ Tregs. However, there was no evidence suggesting SP has any physiological relevance in promoting the rejection of the initial corneal allograft after a second corneal allograft was transplanted onto the other eye.

The crucial role of SP in promoting corneal allograft rejection was demonstrated by the reduced incidence of rejection that occurred when SP was blocked with Spantide II. It remains to be determined if $CD4^+CD25^+$ Tregs are required for sustaining the survival of a corneal allograft, once it has established immune privilege. Although Tregs are essential for the initial acceptance of corneal allografts, their role in sustaining long-term graft survival is less clear.

Cunnusamy et. al. observed that depletion of $CD25^+$ T cells in mice with long-standing corneal allografts did elicit the rejection of the existing corneal allografts [156]. Collectively, the present results, as well as previous studies, suggest that other mechanisms, independent of SP and Tregs, contributed to the rejection of the initial corneal allograft in hosts that received bilateral corneal transplants.

Therapeutic Implications

Currently, the predominant treatment for preventing corneal allograft rejection is the application of topical corticosteroids. However, the administration of steroids increases the risk of developing cataracts and glaucoma. Although routinely used for other forms of solid tissue transplantation, systemic immunosuppressive therapy does not have any further benefit above topical corticosteroids in corneal transplant recipients. Moreover, the long term use of immunosuppressive drugs enhances the risk of malignancy and infection. The development of more specific therapeutic remedies that promote and sustain tolerance with fewer side-effects would be beneficial in preventing the high rejection incidence in corneal allograft patients with pre-disposing factors, such as a previous graft in either eye. Our study gives further insight into the development of future therapies for patients that require multiple corneal transplants, especially in bilateral corneal transplant recipients.

First, by utilizing murine models, we confirmed that hosts requiring multiple corneal transplants are at increased risk of rejecting a subsequent corneal allograft [194, 196, 205, 210, 331]. Patients that rejected a corneal allograft and elect to replace the tissue with another corneal allograft in the same eye are especially at risk. The micro-anatomical environment of the corneal tissue is greatly altered by the initial transplantation procedure, with the ablation of the corneal

nerves and the infiltration of blood and lymphatic vessels, which facilitate activation of the alloimmune response in which ultimately leads to graft rejection. However, in high-risk corneal allograft recipients, topical steroids are not sufficient to prevent rejection and the use of systemic immunosuppression may be required [332, 333]. Cursiefen and colleagues showed in the prevascularized mouse model that blocking both hemangiogenesis and lymphangiogenesis by neutralization of VEGF-A improved graft survival over the controls [187]. Moreover, in a case study on high-risk patients, the anti-VEGF therapy bevacizumab was administered subconjunctivally and perilimbally resulting in 85.7% of the grafts maintaining clarity during the follow-up period [334, 335]. However, we observed in our murine model that destruction of the corneal nerves in one eye can change its local immune privilege status and promote graft rejection. Thus, larger clinical trials are needed to determine if simply blocking angiogenesis will improve graft survival, or if a combination therapy that also modulates the inflammatory response mediated by the destruction of the corneal nerves, would alleviate the high incidence of graft rejection.

Second, the main focus of this study was to determine if bilateral corneal transplant recipients are at higher risk for rejection of the initial, subsequent, or both corneal allografts. We observed that ablation of the corneal nerves in one eye induces an upregulation of SP, making the corneal allograft on the contralateral eye more vulnerable to graft rejection. The release of SP prior to corneal transplantation prevented the induction of Tregs that normally protect corneal allografts, and abolished the suppressive activity of otherwise functional Tregs.

Systemic blockade of SP with the NK-1R antagonist, Spantide II, enhanced graft survival but did not fully restore immune privilege when a graft was transplanted in the other eye 14 days later, possibly due to local perturbations of the ocular environment. It is unknown if topical

application of an NK-1R antagonist would promote graft survival in bilateral corneal transplant recipients. Thus, topical application or subconjunctival injections of an NK-1R antagonist to the subsequent eye might be useful for patients that require bilateral corneal allografts.

Our results suggest that immune privilege can be restored over time, as treating recipients with Spantide II 60 days after the initial corneal nerve ablation gave a better prognosis for graft survival compared to blocking the initial burst of SP at the time the circumferential incision was created. We found that the highest levels of SP were during the early phase after the initial corneal nerve ablation, but over time the level of SP diminished in the contralateral eye, suggesting the level of Spantide II delivered was better at competing for NK-1R than the endogenous SP. However, we did not determine if higher doses of Spantide II at the time of the initial ablation of the corneal nerves would be beneficial in cases where emergency bilateral corneal transplants are needed. Moreover, our results indicated that waiting 100 days after the initial corneal nerve ablation still resulted in a high incidence of graft rejection, but at a slower tempo, suggesting that the alloimmune response may not be as strong due to a partial restoration of immune privilege. These findings are consistent with previous findings in keratoplasty patients indicating that prolonging the time between transplantation of the initial and subsequent corneal allograft improves the survival of both grafts [223].

We observed that the ablation of the corneal nerves in one eye abolishes the established immune privilege of a clear corneal transplant. A simple burst of SP in the ocular environment renders the clear graft vulnerable, and promotes graft rejection. However, our observations suggest that blocking SP does not protect the initial graft from immune rejection when a subsequent corneal allograft is transplanted. This suggests either a more vigorous treatment with

Spantide II is needed to protect the graft from the inflammatory effects of SP, or another immunomodulatory factor is altered and stimulates the rejection of the initial graft.

Lastly, we noted the loss of immune privilege was attributed to SP inhibiting the generation and function of corneal allograft-induced Tregs. Thus, a Treg-based cellular therapy would not be useful if SP activity is high and able to prevent the suppressive activity of otherwise functional Tregs. It would be of future interest to investigate corneal allograft survival in recipients treated with a combination therapy employing an NK-1R antagonist that prevents SP from binding onto Tregs and the administration of *ex vivo*-generated $CD4^+CD25^+$ Tregs.

Future Directions

The information presented in this study demonstrated that the ablation of the corneal nerves from an initial corneal transplant renders both eyes vulnerable corneal allograft rejection. We explored the role of immunomodulatory neuropeptides known to play a pivotal role in ocular immune privilege. However, the ablation of the corneal nerves may affect a myriad of other neuropeptides implicated in the generation of Tregs, such as cortistatin and adrenomodullin [336-338]. Additional experiments will be required to determine what other immunomodulatory neuropeptides found within the eye, such as nerve growth factor [13], brain-derived neurotrophic factor [339], and glial cell-derived neurotrophic factor [14], are important for immune privilege and how a circumferential incision can affect their tolerogenic functions in corneal allograft recipients.

Moreover, this study only addressed the level of SP at the acute phase of neurogenic inflammation within the contralateral eye. We observed that blocking SP 60 days after the initial

cornea incision promoted graft survival, suggesting SP activity mediated the loss of long-standing immune privilege. Moreover, Stein-Streilein observed a retinal-laser burn upregulated NK-1R in the contralateral retina for at least 7 days [69]. Future investigations will elucidate if the long-term loss of immune privilege due to SP activity is the result of sustained upregulation of SP or its receptor, NK-1R.

Our studies noted that SP is increased in the contralateral eye of a circumferentially incised eye, but did not specify what cell type is producing SP. Since SP is quickly upregulated in the contralateral eye after the nerves are ablated, it is likely that a residential cell population, such as the corneal nerves, is producing SP. Thus, the damage induced by the circumferential incision of the subsequent allograft could mediate the release of SP into the corneal tissue and anterior chamber to promote inflammation. Thus, SP may have local ocular inflammatory effects that promote an acute loss of immune privilege. Upon exposure to SP, endothelial cells downregulate pro-apoptotic factors, such as FasL, which permits the survival of $CD4^+$ T cells and macrophages [70, 290]. SP may also promote the antigen presentation function of residential LCs by upregulating MHC II and CD80/CD86 [324, 340, 341], and allowing their infiltration into the cornea. It remains to be determined what cell population is producing SP and how SP is disabling the local immune privilege mechanisms that protect the corneal allograft from rejection.

As stated earlier, an ocular injury in one eye promotes the release of SP within the periphery, and is further upregulated when both eyes are injured. The release of SP within the periphery promotes hematopoiesis of BM progenitor cells into the periphery [291]. Our study noted that once SP is perceived within the peripheral immune system, long-standing immune

privilege is ablated. Our study did not determine which cell populations are functionally altered at the chronic phase of neurogenic inflammation caused by the ablation of the corneal nerves. Future studies will have to determine how a circumferential incision and SP within the venous circulation mediate the loss of long-standing immune privilege.

We observed that SP from a circumferential incision inhibited both the generation and function of $CD4^{+}CD25^{+}$ Tregs. However, our studies did not determine which cell population was directly responding to SP, altering its phenotype to be more inflammatory, and permitting the expression of a robust DTH response. Since both DCs and T cells express NK-1R [302, 312, 326, 327], it remains to be determined which cell populations are directly binding to SP through NK-1R and thus, prevent suppression of the DTH response that leads to graft rejection.

Ablation of the corneal nerves could affect the induction of other forms of tolerance, such as ACAID or oral tolerance. Although these are provocative topics, they are beyond the scope of the current study. Several observations support the notion that ACAID and oral tolerance are intricately linked with the survival of corneal allografts through the induction of antigen-specific tolerance. The induction of either ACAID or oral tolerance prior to corneal transplantation significantly improves the survival of corneal allografts [208, 212, 342-344]. Moreover, many of the manipulations that abolish ACAID also promote corneal allograft rejection [208]. Streilein observed that the ablation of the corneal nerves inhibited the induction of ACAID in the same eye [203], and Stein-Streilein saw that retina-laser burn to one eye prevented the induction of ACAID in the other eye [69]. Thus, additional experiments will need to determine if a circumferential incision can inhibit the induction of ACAID in the contralateral eye, and the induction of peripheral oral tolerance.

Our studies showed a circumferential incision to one eye upregulates both SP and CGRP in the contralateral eye. Although CGRP has been associated with immune privilege of the eye [224], CGRP has also been associated in promoting inflammation in acute spinal cord injury, joint inflammation and inflammatory bowel disease [345-347]. The activation of transient receptor potential ankyrin-repeat 1 (TRPA1) and transient receptor potential vanilloid 1 (TRPV1) on C-reactive nerves promotes the release of both SP and CGRP and induces inflammation [348, 349]. Thus, the co-expression within the corneal nerves and their simultaneous release by TRPA1/TRPV1 activation suggests a collaborative effect between SP and CGRP in promoting the loss of immune privilege. Pre-treatment with the TRPV1 antagonist resiniferatoxin before the development of inflammation renders the neurons less sensitive to noxious stimuli by desensitizing the peripheral afferent nerves and prevents the internalization of TRPV1 receptors [350]. It would be interesting to establish how CGRP was upregulated, and if blocking CGRP or TRPA1/TRPV1activation in bilateral corneal allograft recipients would enhance graft survival.

Corneal allograft-induced $CD4^+CD25^+$ Tregs require FoxP3, CTLA-4, GITR, and m-TGF-β to suppress $CD4^+$ T effector cells *in vitro* [158, 161]. However, our study determined the ablation of the corneal nerves did not affect Treg tolerogenic function by altering the expression levels of these suppressive molecules. This suggests other tolerogenic molecules, such as TRAIL, IL-10 or IL-35 [351, 352], may be responsible for the loss of Treg suppressive function *in vivo*. It remains to be determined what molecules are important for the observed Treg suppressive function *in vivo*, and if different suppressive molecules are differentially regulated in normal risk rejectors and bilateral corneal allograft rejectors.

Lastly, our study noted that an initial corneal allograft hastily underwent rejection after the nerves in the contralateral eye were ablated. Since SP promotes the rejection of an established graft and inhibited the suppressive function of $CD4^+CD25^+$ Tregs, we hypothesized the circumferential incisions promote graft rejection via SP. However, when we administered Spantide II to block SP activity, we did not enhance graft acceptance. It was not determined if the Tregs from the initial corneal allograft were still present and functional after the nerves from the second eye were ablated. Cunnusamy et. al. proposed that the $CD4^+CD25^+$ T cell population is not required for sustaining long-term corneal allograft acceptance [156]. The loss of the Treg population could provide a possible explanation why the initial corneal allograft once again becomes vulnerable to immune mediated rejection. Thus, it remains to be determined why Tregs are not needed for the long term survival of corneal allografts.

References

1. DelMonte, D.W. and T. Kim, *Anatomy and physiology of the cornea.* J Cataract Refract Surg, 2011. **37**(3): p. 588-98.
2. Pinnamaneni, N. and J.L. Funderburgh, *Concise review: Stem cells in the corneal stroma.* Stem Cells, 2012. **30**(6): p. 1059-63.
3. Chang, J.H., et al., *Corneal neovascularization.* Curr Opin Ophthalmol, 2001. **12**(4): p. 242-9.
4. Gupta, D. and C. Illingworth, *Treatments for corneal neovascularization: a review.* Cornea, 2011. **30**(8): p. 927-38.
5. Maddula, S., et al., *Horizons in therapy for corneal angiogenesis.* Ophthalmology, 2011. **118**(3): p. 591-9.
6. Lens, A., S.C. Nemeth, and J.K. Ledford, *Ocular anatomy and physiology*. 2nd ed. 2008, Thorofare, NJ: SLACK. x, 181 p.
7. Muller, L.J., et al., *Corneal nerves: structure, contents and function.* Exp Eye Res, 2003. **76**(5): p. 521-42.
8. Garcia-Hirschfeld, J., L.G. Lopez-Briones, and C. Belmonte, *Neurotrophic influences on corneal epithelial cells.* Exp Eye Res, 1994. **59**(5): p. 597-605.
9. Belmonte, C. and F. Giraldez, *Responses of cat corneal sensory receptors to mechanical and thermal stimulation.* J Physiol, 1981. **321**: p. 355-68.
10. Baker, K.S., et al., *Trigeminal ganglion neurons affect corneal epithelial phenotype. Influence on type VII collagen expression in vitro.* Invest Ophthalmol Vis Sci, 1993. **34**(1): p. 137-44.
11. Chan, K.Y. and R.H. Haschke, *Action of a trophic factor(s) from rabbit corneal epithelial culture on dissociated trigeminal neurons.* J Neurosci, 1981. **1**(10): p. 1155-62.
12. Emoto, I. and R.W. Beuerman, *Stimulation of neurite growth by epithelial implants into corneal stroma.* Neurosci Lett, 1987. **82**(2): p. 140-4.
13. Lambiase, A., et al., *Nerve growth factor promotes corneal healing: structural, biochemical, and molecular analyses of rat and human corneas.* Invest Ophthalmol Vis Sci, 2000. **41**(5): p. 1063-9.
14. You, L., S. Ebner, and F.E. Kruse, *Glial cell-derived neurotrophic factor (GDNF)-induced migration and signal transduction in corneal epithelial cells.* Invest Ophthalmol Vis Sci, 2001. **42**(11): p. 2496-504.
15. Tuominen, I.S., et al., *Corneal innervation and morphology in primary Sjogren's syndrome.* Invest Ophthalmol Vis Sci, 2003. **44**(6): p. 2545-9.
16. Rosenberg, M.E., et al., *In vivo confocal microscopy after herpes keratitis.* Cornea, 2002. **21**(3): p. 265-9.
17. De Cilla, S., et al., *Corneal subbasal nerves changes in patients with diabetic retinopathy: an in vivo confocal study.* Invest Ophthalmol Vis Sci, 2009. **50**(11): p. 5155-8.
18. Niederer, R.L., et al., *Corneal innervation and cellular changes after corneal transplantation: an in vivo confocal microscopy study.* Invest Ophthalmol Vis Sci, 2007. **48**(2): p. 621-6.
19. Darwish, T., et al., *Subbasal nerve fiber regeneration after LASIK and LASEK assessed by noncontact esthesiometry and in vivo confocal microscopy: prospective study.* J Cataract Refract Surg, 2007. **33**(9): p. 1515-21.
20. Ongerboer de Visser, B.W., *Comparative study of corneal and blink reflex latencies in patients with segmental or with cerebral lesions.* Adv Neurol, 1983. **39**: p. 757-72.
21. George, A.J. and D.F. Larkin, *Corneal transplantation: the forgotten graft.* Am J Transplant, 2004. **4**(5): p. 678-85.
22. Armitage, W.J., A.B. Tullo, and D.F. Larkin, *The first successful full-thickness corneal transplant: a commentary on Eduard Zirm's landmark paper of 1906.* Br J Ophthalmol, 2006. **90**(10): p. 1222-3.

23. America, E.B.A.o., *2012 Eye Banking Statistical Report.* EBAA, 2012: p. 1-29.
24. Niederkorn, J.Y., *Cornea: Window to Ocular Immunology.* Curr Immunol Rev. **7**(3): p. 328-335.
25. *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.
26. 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.
27. 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.
28. 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.
29. Kaplan, H.J., T.R. Stevens, and J.W. Streilein, *Transplantation immunology of the anterior chamber of the eye. I. An intra-ocular graft-vs-host reaction (immunogenic anterior uveitis).* J Immunol, 1975. **115**(3): p. 800-4.
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.* J Immunol, 1975. **115**(3): p. 805-10.
31. Niederkorn, J., J.W. Streilein, and J.A. Shadduck, *Deviant immune responses to allogeneic tumors injected intracamerally and subcutaneously in mice.* Invest Ophthalmol Vis Sci, 1981. **20**(3): p. 355-63.
32. Niederkorn, J.Y. and D.F. Larkin, *Immune privilege of corneal allografts.* Ocul Immunol Inflamm. **18**(3): p. 162-71.
33. Hori, J. and J.Y. Niederkorn, *Immunogenicity and immune privilege of corneal allografts.* Chem Immunol Allergy, 2007. **92**: p. 290-9.
34. 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.
35. Skelsey, M.E., J. Mellon, and J.Y. Niederkorn, *Gamma delta T cells are needed for ocular immune privilege and corneal graft survival.* J Immunol, 2001. **166**(7): p. 4327-33.
36. Niederkorn, J.Y., *High-risk corneal allografts and why they lose their immune privilege.* Curr Opin Allergy Clin Immunol. **10**(5): p. 493-7.
37. Sonoda, K.H., M. Taniguchi, and J. Stein-Streilein, *Long-term survival of corneal allografts is dependent on intact CD1d-reactive NKT cells.* J Immunol, 2002. **168**(4): p. 2028-34.
38. Niederkorn, J.Y. and J. Mellon, *Anterior chamber-associated immune deviation promotes corneal allograft survival.* Invest Ophthalmol Vis Sci, 1996. **37**(13): p. 2700-7.
39. Cursiefen, C., et al., *Nonvascular VEGF receptor 3 expression by corneal epithelium maintains avascularity and vision.* Proc Natl Acad Sci U S A, 2006. **103**(30): p. 11405-10.
40. Chen, L., et al., *Vascular endothelial growth factor receptor-3 mediates induction of corneal alloimmunity.* Nat Med, 2004. **10**(8): p. 813-5.
41. 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.
42. Nishida, T. and A.W. Taylor, *Specific aqueous humor factors induce activation of regulatory T cells.* Invest Ophthalmol Vis Sci, 1999. **40**(10): p. 2268-74.
43. D'Orazio, T.J. and J.Y. Niederkorn, *A novel role for TGF-beta and IL-10 in the induction of immune privilege.* J Immunol, 1998. **160**(5): p. 2089-98.
44. Niederkorn, J.Y. and S. Wang, *Immune privilege of the eye and fetus: parallel universes?* Transplantation, 2005. **80**(9): p. 1139-44.

45. Beutelspacher, S.C., et al., *Function of indoleamine 2,3-dioxygenase in corneal allograft rejection and prolongation of allograft survival by over-expression.* Eur J Immunol, 2006. **36**(3): p. 690-700.
46. Lee, D.J. and A.W. Taylor, *Both MC5r and A2Ar are required for protective regulatory immunity in the spleen of post-experimental autoimmune uveitis in mice.* J Immunol, 2013. **191**(8): p. 4103-11.
47. Taylor, A.W. and D. Lee, *Applications of the role of alpha-MSH in ocular immune privilege.* Adv Exp Med Biol, 2010. **681**: p. 143-9.
48. Taylor, A. and K. Namba, *In vitro induction of CD25+ CD4+ regulatory T cells by the neuropeptide alpha-melanocyte stimulating hormone (alpha-MSH).* Immunol Cell Biol, 2001. **79**(4): p. 358-67.
49. Jiang, X., et al., *VIP and growth factors in the infected cornea.* Invest Ophthalmol Vis Sci, 2011. **52**(9): p. 6154-61.
50. Szliter, E.A., et al., *Vasoactive intestinal peptide balances pro- and anti-inflammatory cytokines in the Pseudomonas aeruginosa-infected cornea and protects against corneal perforation.* J Immunol, 2007. **178**(2): p. 1105-14.
51. Berger, E.A., et al., *VIP promotes resistance in the Pseudomonas aeruginosa-infected cornea by modulating adhesion molecule expression.* Invest Ophthalmol Vis Sci, 2010. **51**(11): p. 5776-82.
52. Delgado, M., D. Pozo, and D. Ganea, *The significance of vasoactive intestinal peptide in immunomodulation.* Pharmacol Rev, 2004. **56**(2): p. 249-90.
53. Delgado, M., et al., *Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activation polypeptide (PACAP) protect mice from lethal endotoxemia through the inhibition of TNF-alpha and IL-6.* J Immunol, 1999. **162**(2): p. 1200-5.
54. Jones, M.A. and C.F. Marfurt, *Calcitonin gene-related peptide and corneal innervation: a developmental study in the rat.* J Comp Neurol, 1991. **313**(1): p. 132-50.
55. Stone, R.A. and Y. Kuwayama, *Substance P-like immunoreactive nerves in the human eye.* Arch Ophthalmol, 1985. **103**(8): p. 1207-11.
56. 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.* Invest Ophthalmol Vis Sci, 1998. **39**(8): p. 1372-8.
57. Keen, P., et al., *Substance P in the mouse cornea: effects of chemical and surgical denervation.* Neurosci Lett, 1982. **29**(3): p. 231-5.
58. Denis, P., et al., *Localization and characterization of substance P binding sites in rat and rabbit eyes.* Invest Ophthalmol Vis Sci, 1991. **32**(6): p. 1894-902.
59. Pascual, D.W. and K.L. Bost, *Substance P production by P388D1 macrophages: a possible autocrine function for this neuropeptide.* Immunology, 1990. **71**(1): p. 52-6.
60. Bost, K.L., S.A. Breeding, and D.W. Pascual, *Modulation of the mRNAs encoding substance P and its receptor in rat macrophages by LPS.* Reg Immunol, 1992. **4**(2): p. 105-12.
61. Payan, D.G., *Neuropeptides and inflammation: the role of substance P.* Annu Rev Med, 1989. **40**: p. 341-52.
62. Reid, T.W., et al., *Stimulation of epithelial cell growth by the neuropeptide substance P.* J Cell Biochem, 1993. **52**(4): p. 476-85.
63. Araki-Sasaki, K., et al., *Substance P-induced cadherin expression and its signal transduction in a cloned human corneal epithelial cell line.* J Cell Physiol, 2000. **182**(2): p. 189-95.
64. Nakamura, M., T. Chikama, and T. Nishida, *Up-regulation of integrin alpha 5 expression by combination of substance P and insulin-like growth factor-1 in rabbit corneal epithelial cells.* Biochem Biophys Res Commun, 1998. **246**(3): p. 777-82.

65. Nakamura, M., et al., *Up-regulation of phosphorylation of focal adhesion kinase and paxillin by combination of substance P and IGF-1 in SV-40 transformed human corneal epithelial cells.* Biochem Biophys Res Commun, 1998. **242**(1): p. 16-20.
66. Ziche, M., et al., *Nitric oxide mediates angiogenesis in vivo and endothelial cell growth and migration in vitro promoted by substance P.* J Clin Invest, 1994. **94**(5): p. 2036-44.
67. Braun, A., et al., *Differential modulation of human immunoglobulin isotype production by the neuropeptides substance P, NKA and NKB.* J Neuroimmunol, 1999. **97**(1-2): p. 43-50.
68. Ferguson, T.A., J.D. Hayashi, and H.J. Kaplan, *Regulation of the systemic immune response by visible light and the eye.* FASEB J, 1988. **2**(14): p. 3017-21.
69. Lucas, K., et al., *Retinal laser burn-induced neuropathy leads to substance P-dependent loss of ocular immune privilege.* J Immunol, 2012. **189**(3): p. 1237-42.
70. Ferguson, T.A., et al., *Neuropeptides modulate immune deviation induced via the anterior chamber of the eye.* J Immunol, 1995. **155**(4): p. 1746-56.
71. Hori, J., et al., *GITR ligand-mediated local expansion of regulatory T cells and immune privilege of corneal allografts.* Invest Ophthalmol Vis Sci, 2010. **51**(12): p. 6556-65.
72. Yamada, Y., et al., *Mechanisms of immune suppression for CD8+ T cells by human corneal endothelial cells via membrane-bound TGFbeta.* Invest Ophthalmol Vis Sci, 2010. **51**(5): p. 2548-57.
73. Ferguson, T.A. and T.S. Griffith, *The role of Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL) in the ocular immune response.* Chem Immunol Allergy, 2007. **92**: p. 140-54.
74. Lee, H.O., et al., *TRAIL: a mechanism of tumor surveillance in an immune privileged site.* J Immunol, 2002. **169**(9): p. 4739-44.
75. Hori, J., et al., *B7-H1-induced apoptosis as a mechanism of immune privilege of corneal allografts.* J Immunol, 2006. **177**(9): p. 5928-35.
76. 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.
77. Griffith, T.S., et al., *Fas ligand-induced apoptosis as a mechanism of immune privilege.* Science, 1995. **270**(5239): p. 1189-92.
78. Wang, S., et al., *Role of TRAIL and IFN-gamma in CD4+ T cell-dependent tumor rejection in the anterior chamber of the eye.* J Immunol, 2003. **171**(6): p. 2789-96.
79. Yang, W., et al., *PD-L1 expression on human ocular cells and its possible role in regulating immune-mediated ocular inflammation.* Invest Ophthalmol Vis Sci, 2009. **50**(1): p. 273-80.
80. 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.* Invest Ophthalmol Vis Sci, 2009. **50**(1): p. 263-72.
81. Stuart, P.M., et al., *CD95 ligand (FasL)-induced apoptosis is necessary for corneal allograft survival.* J Clin Invest, 1997. **99**(3): p. 396-402.
82. Yamagami, S., et al., *Role of Fas-Fas ligand interactions in the immunorejection of allogeneic mouse corneal transplants.* Transplantation, 1997. **64**(8): p. 1107-11.
83. Shen, L., et al., *The function of donor versus recipient programmed death-ligand 1 in corneal allograft survival.* J Immunol, 2007. **179**(6): p. 3672-9.
84. Streilein, J.W., J.Y. Niederkorn, and J.A. Shadduck, *Systemic immune unresponsiveness induced in adult mice by anterior chamber presentation of minor histocompatibility antigens.* J Exp Med, 1980. **152**(4): p. 1121-5.
85. 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.

86. 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.
87. 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.* J Immunol, 1978. **120**(3): p. 689-93.
88. 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.
89. Cone, R.E. and R. Pais, *Anterior Chamber-Associated Immune Deviation (ACAID): An Acute Response to Ocular Insult Protects from Future Immune-Mediated Damage?* Ophthalmol Eye Dis, 2009. **1**: p. 33-40.
90. 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.
91. 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.
92. Bellinger, D.L., et al., *Neuropeptide innervation of lymphoid organs.* Ann N Y Acad Sci, 1990. **594**: p. 17-33.
93. Li, X., et al., *The induction of splenic suppressor T cells through an immune-privileged site requires an intact sympathetic nervous system.* J Neuroimmunol, 2004. **153**(1-2): p. 40-9.
94. Skelsey, M.E., E. Mayhew, and J.Y. Niederkorn, *CD25+, interleukin-10-producing CD4+ T cells are required for suppressor cell production and immune privilege in the anterior chamber of the eye.* Immunology, 2003. **110**(1): p. 18-29.
95. Kosiewicz, M.M. and J.W. Streilein, *Intraocular injection of class II-restricted peptide induces an unexpected population of CD8 regulatory cells.* J Immunol, 1996. **157**(5): p. 1905-12.
96. Cone, R.E., S. Chattopadhyay, and J. O'Rourke, *Control of delayed-type hypersensitivity by ocular-induced CD8+ regulatory t cells.* Chem Immunol Allergy, 2008. **94**: p. 138-49.
97. Yamada, J. and J.W. Streilein, *Induction of anterior chamber-associated immune deviation by corneal allografts placed in the anterior chamber.* Invest Ophthalmol Vis Sci, 1997. **38**(13): p. 2833-43.
98. Streilein, J.W., *Ocular immune privilege: therapeutic opportunities from an experiment of nature.* Nat Rev Immunol, 2003. **3**(11): p. 879-89.
99. She, S.C., L.P. Steahly, and E.J. Moticka, *Intracameral injection of allogeneic lymphocytes enhances corneal graft survival.* Invest Ophthalmol Vis Sci, 1990. **31**(10): p. 1950-6.
100. Streilein, J.W. and J.Y. Niederkorn, *Induction of anterior chamber-associated immune deviation requires an intact, functional spleen.* J Exp Med, 1981. **153**(5): p. 1058-67.
101. Niederkorn, J.Y. and D.F. Larkin, *Immune privilege of corneal allografts.* Ocul Immunol Inflamm, 2010. **18**(3): p. 162-71.
102. Williams, K.A. and D.J. Coster, *Penetrating corneal transplantation in the inbred rat: a new model.* Invest Ophthalmol Vis Sci, 1985. **26**(1): p. 23-30.
103. Hamrah, P., et al., *Corneal immunity is mediated by heterogeneous population of antigen-presenting cells.* J Leukoc Biol, 2003. **74**(2): p. 172-8.
104. Niederkorn, J.Y., *Immune mechanisms of corneal allograft rejection.* Curr Eye Res, 2007. **32**(12): p. 1005-16.
105. Teichmann, K.D., *Randomised controlled trial of corticosteroid regimens in endothelial corneal allograft rejection.* Br J Ophthalmol, 2000. **84**(9): p. 1083.
106. Rosenberg, A.S. and A. Singer, *Cellular basis of skin allograft rejection: an in vivo model of immune-mediated tissue destruction.* Annu Rev Immunol, 1992. **10**: p. 333-58.

107. Wang, Y.C., et al., *The influence of MHC and non-MHC genes on the nature of murine cardiac allograft rejection. I. Kinetic analysis of mononuclear cell infiltrate and MHC-class I/class II expression in donor tissue.* Transplantation, 1990. **50**(2): p. 313-24.
108. Sonoda, Y., et al., *Characterization of cell-mediated immune responses elicited by orthotopic corneal allografts in mice.* Invest Ophthalmol Vis Sci, 1995. **36**(2): p. 427-34.
109. Pepose, J.S., et al., *Composition of cellular infiltrates in rejected human corneal allografts.* Graefes Arch Clin Exp Ophthalmol, 1985. **222**(3): p. 128-33.
110. 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.
111. 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.
112. Yamada, J., I. Kurimoto, and J.W. Streilein, *Role of CD4+ T cells in immunobiology of orthotopic corneal transplants in mice.* Invest Ophthalmol Vis Sci, 1999. **40**(11): p. 2614-21.
113. 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.
114. Yamada, J., B.R. Ksander, and J.W. Streilein, *Cytotoxic T cells play no essential role in acute rejection of orthotopic corneal allografts in mice.* Invest Ophthalmol Vis Sci, 2001. **42**(2): p. 386-92.
115. Roy, R., et al., *Pretransplant and posttransplant antibodies in human corneal transplantation.* Transplantation, 1992. **54**(3): p. 463-7.
116. Hahn, A.B., et al., *The association of lymphocytotoxic antibodies with corneal allograft rejection in high risk patients. The Collaborative Corneal Transplantation Studies Research Group.* Transplantation, 1995. **59**(1): p. 21-7.
117. Jager, M.J., et al., *Circulating cornea-specific antibodies in corneal disease and cornea transplantation.* Graefes Arch Clin Exp Ophthalmol, 1994. **232**(2): p. 82-6.
118. Holan, V., et al., *Susceptibility of corneal allografts and xenografts to antibody-mediated rejection.* Immunol Lett, 2005. **100**(2): p. 211-3.
119. Hegde, S., et al., *Effect of alloantibodies on corneal allograft survival.* Invest Ophthalmol Vis Sci, 2002. **43**(4): p. 1012-8.
120. Goslings, W.R., et al., *Corneal transplantation in antibody-deficient hosts.* Invest Ophthalmol Vis Sci, 1999. **40**(1): p. 250-3.
121. Boisgerault, F., et al., *Role of CD4+ and CD8+ T cells in allorecognition: lessons from corneal transplantation.* J Immunol, 2001. **167**(4): p. 1891-9.
122. Cunnusamy, K., P.W. Chen, and J.Y. Niederkorn, *Paradigm shifts in the role of CD4+ T cells in keratoplasty.* Discov Med. **10**(54): p. 452-61.
123. Hegde, S., et al., *CD4(+) T-cell-mediated mechanisms of corneal allograft rejection: role of Fas-induced apoptosis.* Transplantation, 2005. **79**(1): p. 23-31.
124. Haskova, Z., et al., *CD4+ T cells are critical for corneal, but not skin, allograft rejection.* Transplantation, 2000. **69**(4): p. 483-7.
125. Milani, J.K., et al., *Prolongation of corneal allograft survival with liposome-encapsulated cyclosporine in the rat eye.* Ophthalmology, 1993. **100**(6): p. 890-6.
126. 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.
127. Dannenberg, A.M., Jr., *Delayed-type hypersensitivity and cell-mediated immunity in the pathogenesis of tuberculosis.* Immunol Today, 1991. **12**(7): p. 228-33.
128. Turk, J.L., *Relation between delayed hypersensitivity and cell-mediated immunity.* J R Soc Med, 1979. **72**(4): p. 243-5.

129. Huber, B., et al., *Cell-mediated immunity: delayed-type hypersensitivity and cytotoxic responses are mediated by different T-cell subclasses.* J Exp Med, 1976. **143**(6): p. 1534-9.
130. Gell, P.G., *Delayed hypersensitivity: specific cell-mediated immunity.* Br Med Bull, 1967. **23**(1): p. 1-2.
131. Joo, C.K., J.S. Pepose, and P.M. Stuart, *T-cell mediated responses in a murine model of orthotopic corneal transplantation.* Invest Ophthalmol Vis Sci, 1995. **36**(8): p. 1530-40.
132. Torres, P.F., et al., *Cytokine mRNA expression during experimental corneal allograft rejection.* Exp Eye Res, 1996. **63**(4): p. 453-61.
133. Zhu, S., et al., *Early expression of proinflammatory cytokines interleukin-1 and tumor necrosis factor-alpha after corneal transplantation.* J Interferon Cytokine Res, 1999. **19**(6): p. 661-9.
134. Sagoo, P., et al., *Inflammatory cytokines induce apoptosis of corneal endothelium through nitric oxide.* Invest Ophthalmol Vis Sci, 2004. **45**(11): p. 3964-73.
135. Strestikova, P., et al., *FK 506 and aminoguanidine suppress iNOS induction in orthotopic corneal allografts and prolong graft survival in mice.* Nitric Oxide, 2003. **9**(2): p. 111-7.
136. Whitcup, S.M., et al., *Expression of cell adhesion molecules in corneal graft failure.* Cornea, 1993. **12**(6): p. 475-80.
137. Iwata, M., et al., *Mechanisms of lymphocyte adhesion to cultured human corneal epithelial cells.* Curr Eye Res, 1997. **16**(8): p. 751-60.
138. He, Y., et al., *Effect of LFA-1 and ICAM-1 antibody treatment on murine corneal allograft survival.* Invest Ophthalmol Vis Sci, 1994. **35**(8): p. 3218-25.
139. Hori, J., et al., *Specific immunosuppression of corneal allograft rejection by combination of anti-VLA-4 and anti-LFA-1 monoclonal antibodies in mice.* Exp Eye Res, 1997. **65**(1): p. 89-98.
140. Cunnusamy, K., P.W. Chen, and J.Y. Niederkorn, *IL-17 promotes immune privilege of corneal allografts.* J Immunol, 2010. **185**(8): p. 4651-8.
141. Cunnusamy, K., et al., *Two different regulatory T cell populations that promote corneal allograft survival.* Invest Ophthalmol Vis Sci, 2010. **51**(12): p. 6566-74.
142. Hargrave, S.L., et al., *Fate of MHC-matched corneal allografts in Th1-deficient hosts.* Invest Ophthalmol Vis Sci, 2004. **45**(4): p. 1188-93.
143. Cunnusamy, K. and J.Y. Niederkorn, *IFN-gamma blocks CD4+CD25+ Tregs and abolishes immune privilege of minor histocompatibility mismatched corneal allografts.* Am J Transplant, 2013. **13**(12): p. 3076-84.
144. Zhu, J., et al., *GATA-3 promotes Th2 responses through three different mechanisms: induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors.* Cell Res, 2006. **16**(1): p. 3-10.
145. Nicholson, L.B. and V.K. Kuchroo, *Manipulation of the Th1/Th2 balance in autoimmune disease.* Curr Opin Immunol, 1996. **8**(6): p. 837-42.
146. Yamada, J., et al., *Mice with Th2-biased immune systems accept orthotopic corneal allografts placed in "high risk" eyes.* J Immunol, 1999. **162**(9): p. 5247-55.
147. Niederkorn, J.Y., et al., *Allergic airway hyperreactivity increases the risk for corneal allograft rejection.* Am J Transplant, 2009. **9**(5): p. 1017-26.
148. 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.
149. Reyes, N.J., P.W. Chen, and J.Y. Niederkorn, *Allergic Conjunctivitis Renders CD4 T Cells Resistant to T Regulatory Cells and Exacerbates Corneal Allograft Rejection.* Am J Transplant, 2013.
150. 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.

151. Reyes, N.J., P.W. Chen, and J.Y. Niederkorn, *Allergic conjunctivitis renders CD4(+) T cells resistant to t regulatory cells and exacerbates corneal allograft rejection.* Am J Transplant, 2013. **13**(5): p. 1181-92.
152. Korn, T., et al., *IL-17 and Th17 Cells.* Annu Rev Immunol, 2009. **27**: p. 485-517.
153. Muranski, P. and N.P. Restifo, *Essentials of Th17 cell commitment and plasticity.* Blood, 2013. **121**(13): p. 2402-14.
154. Luger, D., et al., *Either a Th17 or a Th1 effector response can drive autoimmunity: conditions of disease induction affect dominant effector category.* J Exp Med, 2008. **205**(4): p. 799-810.
155. Cunnusamy, K., P.W. Chen, and J.Y. Niederkorn, *IL-17 promotes immune privilege of corneal allografts.* J Immunol. **185**(8): p. 4651-8.
156. Cunnusamy, K., P.W. Chen, and J.Y. Niederkorn, *IL-17A-dependent CD4+CD25+ regulatory T cells promote immune privilege of corneal allografts.* J Immunol. **186**(12): p. 6737-45.
157. Chen, H., et al., *A pathogenic role of IL- 17 at the early stage of corneal allograft rejection.* Transpl Immunol, 2009. **21**(3): p. 155-61.
158. Cunnusamy, K., P.W. Chen, and J.Y. Niederkorn, *IL-17A-dependent CD4+CD25+ regulatory T cells promote immune privilege of corneal allografts.* J Immunol, 2011. **186**(12): p. 6737-45.
159. Hori, S., T. Nomura, and S. Sakaguchi, *Control of regulatory T cell development by the transcription factor Foxp3.* Science, 2003. **299**(5609): p. 1057-61.
160. Bluestone, J.A. and A.K. Abbas, *Natural versus adaptive regulatory T cells.* Nat Rev Immunol, 2003. **3**(3): p. 253-7.
161. 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.
162. Linsley, P.S., et al., *CTLA-4 is a second receptor for the B cell activation antigen B7.* J Exp Med, 1991. **174**(3): p. 561-9.
163. Wing, K., et al., *CTLA-4 control over Foxp3+ regulatory T cell function.* Science, 2008. **322**(5899): p. 271-5.
164. Dejean, A.S., et al., *Transcription factor Foxo3 controls the magnitude of T cell immune responses by modulating the function of dendritic cells.* Nat Immunol, 2009. **10**(5): p. 504-13.
165. Onishi, Y., et al., *Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation.* Proc Natl Acad Sci U S A, 2008. **105**(29): p. 10113-8.
166. Munn, D.H., et al., *Inhibition of T cell proliferation by macrophage tryptophan catabolism.* J Exp Med, 1999. **189**(9): p. 1363-72.
167. Kanamaru, F., et al., *Costimulation via glucocorticoid-induced TNF receptor in both conventional and CD25+ regulatory CD4+ T cells.* J Immunol, 2004. **172**(12): p. 7306-14.
168. Yu, K.Y., et al., *Identification of a ligand for glucocorticoid-induced tumor necrosis factor receptor constitutively expressed in dendritic cells.* Biochem Biophys Res Commun, 2003. **310**(2): p. 433-8.
169. Tone, M., et al., *Mouse glucocorticoid-induced tumor necrosis factor receptor ligand is costimulatory for T cells.* Proc Natl Acad Sci U S A, 2003. **100**(25): p. 15059-64.
170. Kehrl, J.H., et al., *Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth.* J Exp Med, 1986. **163**(5): p. 1037-50.
171. Chen, W., et al., *Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3.* J Exp Med, 2003. **198**(12): p. 1875-86.
172. Gorelik, L., S. Constant, and R.A. Flavell, *Mechanism of transforming growth factor beta-induced inhibition of T helper type 1 differentiation.* J Exp Med, 2002. **195**(11): p. 1499-505.
173. Arentsen, J.J., *Corneal transplant allograft reaction: possible predisposing factors.* Trans Am Ophthalmol Soc, 1983. **81**: p. 361-402.

174. Yamagami, S., Y. Suzuki, and T. Tsuru, *Risk factors for graft failure in penetrating keratoplasty.* Acta Ophthalmol Scand, 1996. **74**(6): p. 584-8.
175. Bachmann, B., R.S. Taylor, and C. Cursiefen, *Corneal neovascularization as a risk factor for graft failure and rejection after keratoplasty: an evidence-based meta-analysis.* Ophthalmology, 2010. **117**(7): p. 1300-5 e7.
176. Coster, D.J. and K.A. Williams, *The impact of corneal allograft rejection on the long-term outcome of corneal transplantation.* Am J Ophthalmol, 2005. **140**(6): p. 1112-22.
177. Hargrave, S., et al., *Preliminary findings in corneal allograft rejection in patients with keratoconus.* Am J Ophthalmol, 2003. **135**(4): p. 452-60.
178. Kuchle, M., et al., *Risk factors for corneal allograft rejection: intermediate results of a prospective normal-risk keratoplasty study.* Graefes Arch Clin Exp Ophthalmol, 2002. **240**(7): p. 580-4.
179. Larkin, D.F., *Corneal transplantation for herpes simplex keratitis.* Br J Ophthalmol, 1998. **82**(2): p. 107-8.
180. Regenfuss, B., F. Bock, and C. Cursiefen, *Corneal angiogenesis and lymphangiogenesis.* Curr Opin Allergy Clin Immunol, 2012. **12**(5): p. 548-54.
181. Albuquerque, R.J., et al., *Alternatively spliced vascular endothelial growth factor receptor-2 is an essential endogenous inhibitor of lymphatic vessel growth.* Nat Med, 2009. **15**(9): p. 1023-30.
182. Cursiefen, C., et al., *Corneal lymphangiogenesis: evidence, mechanisms, and implications for corneal transplant immunology.* Cornea, 2003. **22**(3): p. 273-81.
183. Bachmann, B.O., et al., *Promotion of graft survival by vascular endothelial growth factor a neutralization after high-risk corneal transplantation.* Arch Ophthalmol, 2008. **126**(1): p. 71-7.
184. 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.
185. Sano, Y., B.R. Ksander, and J.W. Streilein, *Fate of orthotopic corneal allografts in eyes that cannot support anterior chamber-associated immune deviation induction.* Invest Ophthalmol Vis Sci, 1995. **36**(11): p. 2176-85.
186. Chen, L., et al., *Vascular endothelial growth factor receptor-3 mediates induction of corneal alloimmunity. 2004.* Ocul Immunol Inflamm, 2007. **15**(3): p. 275-8.
187. Cursiefen, C., et al., *Inhibition of hemangiogenesis and lymphangiogenesis after normal-risk corneal transplantation by neutralizing VEGF promotes graft survival.* Invest Ophthalmol Vis Sci, 2004. **45**(8): p. 2666-73.
188. Dietrich, T., et al., *Cutting edge: lymphatic vessels, not blood vessels, primarily mediate immune rejections after transplantation.* J Immunol, 2010. **184**(2): p. 535-9.
189. 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.
190. Ghoraishi, M., et al., *Penetrating keratoplasty in atopic keratoconjunctivitis.* Cornea, 1995. **14**(6): p. 610-3.
191. Flynn, T.H., et al., *The effect of perioperative allergic conjunctivitis on corneal lymphangiogenesis after corneal transplantation.* Br J Ophthalmol, 2011. **95**(10): p. 1451-6.
192. Flynn, T.H., et al., *Effect of allergic conjunctival inflammation on the allogeneic response to donor cornea.* Invest Ophthalmol Vis Sci, 2007. **48**(9): p. 4044-9.
193. Claesson, M. and W.J. Armitage, *Clinical outcome of repeat penetrating keratoplasty.* Cornea, 2013. **32**(7): p. 1026-30.
194. Al-Mezaine, H., M.D. Wagoner, and G. King Khaled Eye Specialist Hospital Cornea Transplant Study, *Repeat penetrating keratoplasty: indications, graft survival, and visual outcome.* Br J Ophthalmol, 2006. **90**(3): p. 324-7.
195. America, E.B.A.o., *1992 Eye Banking Statistical Report.* EBAA, 1992: p. 1-30.

196. Weisbrod, D.J., et al., *Outcomes of repeat penetrating keratoplasty and risk factors for graft failure.* Cornea, 2003. **22**(5): p. 429-34.
197. Braude, L.S. and J.W. Chandler, *Corneal allograft rejection. The role of the major histocompatibility complex.* Surv Ophthalmol, 1983. **27**(5): p. 290-305.
198. Nicholls, S.M., B.B. Bradley, and D.L. Easty, *Effect of mismatches for major histocompatibility complex and minor antigens on corneal graft rejection.* Invest Ophthalmol Vis Sci, 1991. **32**(10): p. 2729-34.
199. Sano, Y., B.R. Ksander, and J.W. Streilein, *Murine orthotopic corneal transplantation in high-risk eyes. Rejection is dictated primarily by weak rather than strong alloantigens.* Invest Ophthalmol Vis Sci, 1997. **38**(6): p. 1130-8.
200. Fink, N., et al., *Effectiveness of histocompatibility matching in high-risk corneal transplantation: a summary of results from the Collaborative Corneal Transplantation Studies.* Cesk Oftalmol, 1994. **50**(1): p. 3-12.
201. Khodadoust, A.A. and A.M. Silverstein, *Studies on the nature of the privilege enjoyed by corneal allografts.* Invest Ophthalmol, 1972. **11**(3): p. 137-48.
202. Streilein, J.W., C. Arancibia-Caracamo, and H. Osawa, *The role of minor histocompatibility alloantigens in penetrating keratoplasty.* Dev Ophthalmol, 2003. **36**: p. 74-88.
203. Streilein, J.W., et al., *Immunosuppressive properties of tissues obtained from eyes with experimentally manipulated corneas.* Invest Ophthalmol Vis Sci, 1996. **37**(2): p. 413-24.
204. Maumenee, A.E., *The immune concept: its relation to corneal homotransplantation.* Ann N Y Acad Sci, 1955. **59**(3): p. 453-61.
205. Patel, N.P., et al., *Indications for and outcomes of repeat penetrating keratoplasty, 1989-1995.* Ophthalmology, 2000. **107**(4): p. 719-24.
206. Rapuano, C.J., et al., *Indications for and outcomes of repeat penetrating keratoplasty.* Am J Ophthalmol, 1990. **109**(6): p. 689-95.
207. Dana, M.R., *Angiogenesis and lymphangiogenesis-implications for corneal immunity.* Semin Ophthalmol, 2006. **21**(1): p. 19-22.
208. Niederkorn, J.Y., *Corneal transplantation and immune privilege.* Int Rev Immunol, 2013. **32**(1): p. 57-67.
209. Niederkorn, J.Y., *The immune privilege of corneal allografts.* Transplantation, 1999. **67**(12): p. 1503-8.
210. Robinson, C.H., *Indications, complications and prognosis for repeat penetrating keratoplasty.* Ophthalmic Surg, 1979. **10**(5): p. 27-34.
211. Ashour, H.M. and J.Y. Niederkorn, *Peripheral tolerance via the anterior chamber of the eye: role of B cells in MHC class I and II antigen presentation.* J Immunol, 2006. **176**(10): p. 5950-7.
212. He, Y.G., J. Mellon, and J.Y. Niederkorn, *The effect of oral immunization on corneal allograft survival.* Transplantation, 1996. **61**(6): p. 920-6.
213. Magone, M.T., et al., *A novel murine model of allergic conjunctivitis.* Clin Immunol Immunopathol, 1998. **87**(1): p. 75-84.
214. Chu, D.S. and C.S. Foster, *Sympathetic ophthalmia.* Int Ophthalmol Clin, 2002. **42**(3): p. 179-85.
215. Chaithanyaa, N., et al., *Sympathetic ophthalmia: a review of literature.* Oral Surg Oral Med Oral Pathol Oral Radiol, 2012. **113**(2): p. 172-6.
216. Caspi, R.R., *Ocular autoimmunity: the price of privilege?* Immunol Rev, 2006. **213**: p. 23-35.
217. Gery, I. and J.W. Streilein, *Autoimmunity in the eye and its regulation.* Curr Opin Immunol, 1994. **6**(6): p. 938-45.
218. Khodadoust, A.A. and Y. Karnema, *Corneal grafts in the second eye.* Cornea, 1984. **3**(1): p. 17-20.
219. Donshik, P.C., et al., *Effect of bilateral and unilateral grafts on the incidence of rejections in keratoconus.* Am J Ophthalmol, 1979. **87**(6): p. 823-6.

220. Meyer, R.F., *Corneal allograft rejection in bilateral penetrating keratoplasty: clinical and laboratory studies.* Trans Am Ophthalmol Soc, 1986. **84**: p. 664-742.
221. Buxton, J.N., M. Schuman, and J. Pecego, *Graft reactions after unilateral and bilateral keratoplasty for keratoconus.* Ophthalmology, 1981. **88**(8): p. 771-3.
222. Malbran, E.S. and R.E. Fernandez-Meijide, *Bilateral versus unilateral penetrating graft in keratoconus.* Ophthalmology, 1982. **89**(1): p. 38-40.
223. Tuft, S.J., W.M. Gregory, and C.R. Davison, *Bilateral penetrating keratoplasty for keratoconus.* Ophthalmology, 1995. **102**(3): p. 462-8.
224. Streilein, J.W., et al., *Neural control of ocular immune privilege.* Ann N Y Acad Sci, 2000. **917**: p. 297-306.
225. Taylor, A.W. and N. Kitaichi, *The diminishment of experimental autoimmune encephalomyelitis (EAE) by neuropeptide alpha-melanocyte stimulating hormone (alpha-MSH) therapy.* Brain Behav Immun, 2008. **22**(5): p. 639-46.
226. Yamada, J., et al., *Allogeneic corneal tolerance in rodents with long-term graft survival.* Transplantation, 2005. **79**(10): p. 1362-9.
227. Niederkorn, J.Y., *High-risk corneal allografts and why they lose their immune privilege.* Curr Opin Allergy Clin Immunol.
228. Bersudsky, V., et al., *The profile of repeated corneal transplantation.* Ophthalmology, 2001. **108**(3): p. 461-9.
229. Ensley, R.D., et al., *Predictors of survival after repeat heart transplantation. The Registry of the International Society for Heart and Lung Transplantation, and Contributing Investigators.* J Heart Lung Transplant, 1992. **11**(3 Pt 2): p. S142-58.
230. Taylor, C.J. and P.A. Dyer, *Histocompatibility antigens.* Eye (Lond), 1995. **9 (Pt 2)**: p. 173-9.
231. Gavrilov, J.C., et al., *Influencing factors on the suitability of organ-cultured corneas.* Eye (Lond), 2010. **24**(7): p. 1227-33.
232. Armitage, W.J., et al., *The suitability of corneas stored by organ culture for penetrating keratoplasty and influence of donor and recipient factors on 5-year graft survival.* Invest Ophthalmol Vis Sci, 2014. **55**(2): p. 784-91.
233. Banerjee, S., A.D. Dick, and S.M. Nicholls, *Factors affecting rejection of second corneal transplants in rats.* Transplantation, 2004. **77**(4): p. 492-6.
234. Krummey, S.M. and M.L. Ford, *Heterogeneity within T Cell Memory: Implications for Transplant Tolerance.* Front Immunol, 2012. **3**: p. 36.
235. Welsh, R.M., L.K. Selin, and E. Szomolanyi-Tsuda, *Immunological memory to viral infections.* Annu Rev Immunol, 2004. **22**: p. 711-43.
236. Petrova, G., A. Ferrante, and J. Gorski, *Cross-reactivity of T cells and its role in the immune system.* Crit Rev Immunol, 2012. **32**(4): p. 349-72.
237. Adams, A.B., et al., *Heterologous immunity provides a potent barrier to transplantation tolerance.* J Clin Invest, 2003. **111**(12): p. 1887-95.
238. Welsh, R.M. and L.K. Selin, *No one is naive: the significance of heterologous T-cell immunity.* Nat Rev Immunol, 2002. **2**(6): p. 417-26.
239. Caspi, R.R., *A look at autoimmunity and inflammation in the eye.* J Clin Invest, 2010. **120**(9): p. 3073-83.
240. Rao, N.A., *Mechanisms of inflammatory response in sympathetic ophthalmia and VKH syndrome.* Eye (Lond), 1997. **11 (Pt 2)**: p. 213-6.
241. Rao, N.A. and V.G. Wong, *Aetiology of sympathetic ophthalmitis.* Trans Ophthalmol Soc U K, 1981. **101 (Pt 3)**(3): p. 357-60.
242. Sen, H.N. and R.B. Nussenblatt, *Sympathetic ophthalmia: what have we learned?* Am J Ophthalmol, 2009. **148**(5): p. 632-3.

243. Maumenee, A.E., *Clinical aspects of the corneal homograft reaction.* Invest Ophthalmol, 1962. **1**: p. 244-52.
244. Sharma, N., et al., *Penetrating autokeratoplasty for unilateral corneal opacification.* Eye Contact Lens, 2012. **38**(2): p. 112-5.
245. Arnalich-Montiel, F. and J.K. Dart, *Ipsilateral rotational autokeratoplasty: a review.* Eye (Lond), 2009. **23**(10): p. 1931-8.
246. Price, F.W., Jr. and S.I. Hanna, *Bilateral penetrating autokeratoplasty.* J Refract Surg, 1995. **11**(6): p. 494-6.
247. Paque, J. and R.H. Poirier, *Corneal allograft reaction and its relationship to suture site neovascularization.* Ophthalmic Surg, 1977. **8**(4): p. 71-4.
248. Tchah, H., D.H. Youn, and E.J. Holland, *Experimental orthotopic penetrating keratoplasty--a rat penetrating keratoplasty model.* J Korean Med Sci, 1991. **6**(1): p. 15-9.
249. Dana, M.R. and J.W. Streilein, *Loss and restoration of immune privilege in eyes with corneal neovascularization.* Invest Ophthalmol Vis Sci, 1996. **37**(12): p. 2485-94.
250. 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.
251. Hajrasouliha, A.R., et al., *Vascular endothelial growth factor-C promotes alloimmunity by amplifying antigen-presenting cell maturation and lymphangiogenesis.* Invest Ophthalmol Vis Sci, 2012. **53**(3): p. 1244-50.
252. Patel, S.P. and R. Dana, *Corneal lymphangiogenesis: implications in immunity.* Semin Ophthalmol, 2009. **24**(3): p. 135-8.
253. Chung, E.S., et al., *Regulation of blood vessel versus lymphatic vessel growth in the cornea.* Invest Ophthalmol Vis Sci, 2009. **50**(4): p. 1613-8.
254. Chung, E.S., et al., *Contribution of macrophages to angiogenesis induced by vascular endothelial growth factor receptor-3-specific ligands.* Am J Pathol, 2009. **175**(5): p. 1984-92.
255. Cursiefen, C., et al., *VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment.* J Clin Invest, 2004. **113**(7): p. 1040-50.
256. Perez-Santonja, J.J., et al., *Corneal sensitivity after photorefractive keratectomy and laser in situ keratomileusis for low myopia.* Am J Ophthalmol, 1999. **127**(5): p. 497-504.
257. Albietz, J.M. and L.M. Lenton, *Management of the ocular surface and tear film before, during, and after laser in situ keratomileusis.* J Refract Surg, 2004. **20**(1): p. 62-71.
258. Erie, J.C., et al., *Recovery of corneal subbasal nerve density after PRK and LASIK.* Am J Ophthalmol, 2005. **140**(6): p. 1059-1064.
259. Wilson, S.E. and R. Ambrosio, *Laser in situ keratomileusis-induced neurotrophic epitheliopathy.* Am J Ophthalmol, 2001. **132**(3): p. 405-6.
260. Ambrosio, R., Jr., T. Tervo, and S.E. Wilson, *LASIK-associated dry eye and neurotrophic epitheliopathy: pathophysiology and strategies for prevention and treatment.* J Refract Surg, 2008. **24**(4): p. 396-407.
261. Nettune, G.R. and S.C. Pflugfelder, *Post-LASIK tear dysfunction and dysesthesia.* Ocul Surf, 2010. **8**(3): p. 135-45.
262. O'Brien, T.P., et al., *Inflammatory response in the early stages of wound healing after excimer laser keratectomy.* Arch Ophthalmol, 1998. **116**(11): p. 1470-4.
263. Nishida, T., *Corneal Healing Responses to Injuries and Refractive Surgeries.* 1998: p. 139.
264. Cubitt, C.L., et al., *IL-8 gene expression in cultures of human corneal epithelial cells and keratocytes.* Invest Ophthalmol Vis Sci, 1993. **34**(11): p. 3199-206.

265. Shankar, G., S. Pickard-Elias, and K. Burnham, *Superantigen-induced Langerhans cell depletion is mediated by epidermal cell-derived IL-1 alpha and TNF alpha.* Cell Immunol, 1996. **171**(2): p. 240-5.
266. Gao, N., et al., *Dendritic cell-epithelium interplay is a determinant factor for corneal epithelial wound repair.* Am J Pathol, 2011. **179**(5): p. 2243-53.
267. Oh, J.Y., et al., *Analysis of macrophage phenotype in rejected corneal allografts.* Invest Ophthalmol Vis Sci, 2013. **54**(12): p. 7779-84.
268. Ebihara, N., et al., *Role of the IL-6 classic- and trans-signaling pathways in corneal sterile inflammation and wound healing.* Invest Ophthalmol Vis Sci, 2011. **52**(12): p. 8549-57.
269. Hamrah, P., et al., *Unilateral herpes zoster ophthalmicus results in bilateral corneal nerve alteration: an in vivo confocal microscopy study.* Ophthalmology, 2013. **120**(1): p. 40-7.
270. Nagasato, D., et al., *Morphological changes of corneal subepithelial nerve plexus in different types of herpetic keratitis.* Jpn J Ophthalmol, 2011. **55**(5): p. 444-50.
271. Hamrah, P., et al., *Corneal sensation and subbasal nerve alterations in patients with herpes simplex keratitis: an in vivo confocal microscopy study.* Ophthalmology, 2010. **117**(10): p. 1930-6.
272. Vega, J.L., H. Keino, and S. Masli, *Surgical denervation of ocular sympathetic afferents decreases local transforming growth factor-beta and abolishes immune privilege.* Am J Pathol, 2009. **175**(3): p. 1218-25.
273. Hutchins, B., et al., *Calcitonin gene-related peptide and substance P immunoreactivity in rat trigeminal ganglia and brainstem following adjuvant-induced inflammation of the temporomandibular joint.* Arch Oral Biol, 2000. **45**(4): p. 335-45.
274. Decaris, E., et al., *Evidence for neurogenic transmission inducing degenerative cartilage damage distant from local inflammation.* Arthritis Rheum, 1999. **42**(9): p. 1951-60.
275. Matsuo, T., J. Suzuki, and F. Shiraga, *Aqueous flare elevation in the fellow eye after vitrectomy.* Ophthalmic Surg Lasers, 2000. **31**(4): p. 282-6.
276. El-Harazi, S.M., et al., *Consensual inflammation following ocular surgery.* Ophthalmic Surg Lasers, 1999. **30**(4): p. 254-9.
277. Tomas-Barberan, S. and P. Fagerholm, *Anterior chamber flare after photorefractive keratectomy.* J Refract Surg, 1996. **12**(1): p. 103-7.
278. Vita, R.C., et al., *Alterations in blood-aqueous barrier after corneal refractive surgery.* Cornea, 1998. **17**(2): p. 158-62.
279. El-Harazi, S.M., A.Z. Chuang, and R.W. Yee, *Assessment of anterior chamber flare and cells after laser in situ keratomileusis.* J Cataract Refract Surg, 2001. **27**(5): p. 693-6.
280. Hamrah, P., et al., *Local treatment with alpha-melanocyte stimulating hormone reduces corneal allorejection.* Transplantation, 2009. **88**(2): p. 180-7.
281. Taylor, A.W. and D.J. Lee, *The alpha-melanocyte stimulating hormone induces conversion of effector T cells into treg cells.* J Transplant, 2011. **2011**: p. 246856.
282. Ozbek, Z., et al., *Graft rejection risk and incidence after bilateral penetrating keratoplasty.* Eye Contact Lens, 2008. **34**(3): p. 174-8.
283. Ziche, M., et al., *Substance P stimulates neovascularization in vivo and proliferation of cultured endothelial cells.* Microvasc Res, 1990. **40**(2): p. 264-78.
284. Xu, X.J., et al., *NK-1, but not NK-2, tachykinin receptors mediate plasma extravasation induced by antidromic C-fiber stimulation in rat hindpaw: demonstrated with the NK-1 antagonist CP-96,345 and the NK-2 antagonist Men 10207.* Neurosci Lett, 1992. **139**(2): p. 249-52.
285. Hamrah, P., et al., *Deletion of the chemokine receptor CCR1 prolongs corneal allograft survival.* Invest Ophthalmol Vis Sci, 2007. **48**(3): p. 1228-36.

286. Yamagami, S., et al., *Differential chemokine gene expression in corneal transplant rejection.* Invest Ophthalmol Vis Sci, 1999. **40**(12): p. 2892-7.
287. Sun, J. and M. Bhatia, *Blockade of neurokinin-1 receptor attenuates CC and CXC chemokine production in experimental acute pancreatitis and associated lung injury.* Am J Physiol Gastrointest Liver Physiol, 2007. **292**(1): p. G143-53.
288. Dudek, H., et al., *Regulation of neuronal survival by the serine-threonine protein kinase Akt.* Science, 1997. **275**(5300): p. 661-5.
289. Ferguson, T.A. and T.S. Griffith, *A vision of cell death: insights into immune privilege.* Immunol Rev, 1997. **156**: p. 167-84.
290. Zhou, Z., et al., *Substance P delays apoptosis, enhancing keratitis after Pseudomonas aeruginosa infection.* Invest Ophthalmol Vis Sci, 2008. **49**(10): p. 4458-67.
291. Hong, H.S., et al., *A new role of substance P as an injury-inducible messenger for mobilization of CD29(+) stromal-like cells.* Nat Med, 2009. **15**(4): p. 425-35.
292. Imai, S., et al., *Calcitonin gene-related peptide, substance P, and tyrosine hydroxylase-immunoreactive innervation of rat bone marrows: an immunohistochemical and ultrastructural investigation on possible efferent and afferent mechanisms.* J Orthop Res, 1997. **15**(1): p. 133-40.
293. Kang, H.S., et al., *Neurokinin receptors: relevance to the emerging immune system.* Arch Immunol Ther Exp (Warsz), 2004. **52**(5): p. 338-47.
294. Rameshwar, P., *Substance P: a regulatory neuropeptide for hematopoiesis and immune functions.* Clin Immunol Immunopathol, 1997. **85**(2): p. 129-33.
295. Calvo, W., *The innervation of the bone marrow in laboratory animals.* Am J Anat, 1968. **123**(2): p. 315-28.
296. Takase, B. and S. Nomura, *Studies on the innervation of the bone marrow.* J Comp Neurol, 1957. **108**(3): p. 421-43.
297. Rameshwar, P., et al., *Receptor induction regulates the synergistic effects of substance P with IL-1 and platelet-derived growth factor on the proliferation of bone marrow fibroblasts.* J Immunol, 1997. **158**(7): p. 3417-24.
298. Rameshwar, P., A. Poddar, and P. Gascon, *Hematopoietic regulation mediated by interactions among the neurokinins and cytokines.* Leuk Lymphoma, 1997. **28**(1-2): p. 1-10.
299. Moore, R.N., et al., *Substance P augmentation of CSF-1-stimulated in vitro myelopoiesis. A two-signal progenitor restricted, tuftsin-like effect.* J Immunol, 1988. **141**(8): p. 2699-703.
300. Rameshwar, P., et al., *Substance p-fibronectin-cytokine interactions in myeloproliferative disorders with bone marrow fibrosis.* Acta Haematol, 2003. **109**(1): p. 1-10.
301. Amadesi, S., et al., *Role for substance p-based nociceptive signaling in progenitor cell activation and angiogenesis during ischemia in mice and in human subjects.* Circulation, 2012. **125**(14): p. 1774-86, S1-19.
302. Maggi, C.A., *The effects of tachykinins on inflammatory and immune cells.* Regul Pept, 1997. **70**(2-3): p. 75-90.
303. Rameshwar, P. and P. Gascon, *Substance P (SP) mediates production of stem cell factor and interleukin-1 in bone marrow stroma: potential autoregulatory role for these cytokines in SP receptor expression and induction.* Blood, 1995. **86**(2): p. 482-90.
304. Honor, P., et al., *Spinal substance P receptor expression and internalization in acute, short-term, and long-term inflammatory pain states.* J Neurosci, 1999. **19**(17): p. 7670-8.
305. McCormack, R.J., R.P. Hart, and D. Ganea, *Expression of NK-1 receptor mRNA in murine T lymphocytes.* Neuroimmunomodulation, 1996. **3**(1): p. 35-46.
306. Payan, D.G., et al., *Substance P recognition by a subset of human T lymphocytes.* J Clin Invest, 1984. **74**(4): p. 1532-9.

307. Streilein, J.W., *New thoughts on the immunology of corneal transplantation.* Eye (Lond), 2003. **17**(8): p. 943-8.
308. Payan, D.G., J.D. Levine, and E.J. Goetzl, *Modulation of immunity and hypersensitivity by sensory neuropeptides.* J Immunol, 1984. **132**(4): p. 1601-4.
309. Stanisz, A.M., D. Befus, and J. Bienenstock, *Differential effects of vasoactive intestinal peptide, substance P, and somatostatin on immunoglobulin synthesis and proliferations by lymphocytes from Peyer's patches, mesenteric lymph nodes, and spleen.* J Immunol, 1986. **136**(1): p. 152-6.
310. Payan, D.G., D.R. Brewster, and E.J. Goetzl, *Specific stimulation of human T lymphocytes by substance P.* J Immunol, 1983. **131**(4): p. 1613-5.
311. Lambrecht, B.N., et al., *Endogenously produced substance P contributes to lymphocyte proliferation induced by dendritic cells and direct TCR ligation.* Eur J Immunol, 1999. **29**(12): p. 3815-25.
312. Lai, J.P., S.D. Douglas, and W.Z. Ho, *Human lymphocytes express substance P and its receptor.* J Neuroimmunol, 1998. **86**(1): p. 80-6.
313. Nio, D.A., R.N. Moylan, and J.K. Roche, *Modulation of T lymphocyte function by neuropeptides. Evidence for their role as local immunoregulatory elements.* J Immunol, 1993. **150**(12): p. 5281-8.
314. Calvo, C.F., G. Chavanel, and A. Senik, *Substance P enhances IL-2 expression in activated human T cells.* J Immunol, 1992. **148**(11): p. 3498-504.
315. Niederkorn, J.Y., et al., *Allergic conjunctivitis exacerbates corneal allograft rejection by activating Th1 and th2 alloimmune responses.* J Immunol. **184**(11): p. 6076-83.
316. Kawamura, N., et al., *Differential effects of neuropeptides on cytokine production by mouse helper T cell subsets.* Neuroimmunomodulation, 1998. **5**(1-2): p. 9-15.
317. Cunin, P., et al., *The tachykinins substance P and hemokinin-1 favor the generation of human memory Th17 cells by inducing IL-1beta, IL-23, and TNF-like 1A expression by monocytes.* J Immunol, 2011. **186**(7): p. 4175-82.
318. Dwyer, K.M., et al., *Expression of CD39 by human peripheral blood CD4+ CD25+ T cells denotes a regulatory memory phenotype.* Am J Transplant, 2010. **10**(11): p. 2410-20.
319. Grant, C.R., et al., *Dysfunctional CD39(POS) regulatory T cells and aberrant control of T-helper type 17 cells in autoimmune hepatitis.* Hepatology, 2014. **59**(3): p. 1007-15.
320. Fletcher, J.M., et al., *CD39+Foxp3+ regulatory T Cells suppress pathogenic Th17 cells and are impaired in multiple sclerosis.* J Immunol, 2009. **183**(11): p. 7602-10.
321. Loza, M.J., et al., *T-cell specific defect in expression of the NTPDase CD39 as a biomarker for lupus.* Cell Immunol, 2011. **271**(1): p. 110-7.
322. Koli, K., et al., *Latency, activation, and binding proteins of TGF-beta.* Microsc Res Tech, 2001. **52**(4): p. 354-62.
323. Khalil, N., *TGF-beta: from latent to active.* Microbes Infect, 1999. **1**(15): p. 1255-63.
324. Marriott, I., et al., *Substance P activates NF-kappaB independent of elevations in intracellular calcium in murine macrophages and dendritic cells.* J Neuroimmunol, 2000. **102**(2): p. 163-71.
325. Fiebich, B.L., et al., *The neuropeptide substance P activates p38 mitogen-activated protein kinase resulting in IL-6 expression independently from NF-kappa B.* J Immunol, 2000. **165**(10): p. 5606-11.
326. Janelsins, B.M., et al., *Neurokinin-1 receptor agonists bias therapeutic dendritic cells to induce type 1 immunity by licensing host dendritic cells to produce IL-12.* Blood, 2013. **121**(15): p. 2923-33.
327. Mathers, A.R., et al., *In vivo signaling through the neurokinin 1 receptor favors transgene expression by Langerhans cells and promotes the generation of Th1- and Tc1-biased immune responses.* J Immunol, 2007. **178**(11): p. 7006-17.

328. Janelsins, B.M., et al., *Proinflammatory tachykinins that signal through the neurokinin 1 receptor promote survival of dendritic cells and potent cellular immunity.* Blood, 2009. **113**(13): p. 3017-26.
329. Williams, K.A., et al., *The influence of rejection episodes in recipients of bilateral corneal grafts.* Am J Transplant, 2010. **10**(4): p. 921-30.
330. Sakaguchi, S., et al., *The plasticity and stability of regulatory T cells.* Nat Rev Immunol, 2013. **13**(6): p. 461-7.
331. Insler, M.S. and B. Pechous, *Visual results in repeat penetrating keratoplasty.* Am J Ophthalmol, 1986. **102**(3): p. 371-5.
332. Hill, J.C., *Systemic cyclosporine in high-risk keratoplasty: long-term results.* Eye (Lond), 1995. **9 (Pt 4)**: p. 422-8.
333. Joseph, A., et al., *Tacrolimus immunosuppression in high-risk corneal grafts.* Br J Ophthalmol, 2007. **91**(1): p. 51-5.
334. Vassileva, P.I. and T.G. Hergeldzhieva, *Avastin use in high risk corneal transplantation.* Graefes Arch Clin Exp Ophthalmol, 2009. **247**(12): p. 1701-6.
335. Symes, R.J. and T.R. Poole, *Corneal graft surgery combined with subconjunctival bevacizumab (avastin).* Cornea, 2010. **29**(6): p. 691-3.
336. Ganea, D., E. Gonzalez-Rey, and M. Delgado, *A novel mechanism for immunosuppression: from neuropeptides to regulatory T cells.* J Neuroimmune Pharmacol, 2006. **1**(4): p. 400-9.
337. Souza-Moreira, L., et al., *Neuropeptides as pleiotropic modulators of the immune response.* Neuroendocrinology, 2011. **94**(2): p. 89-100.
338. Wang, J., et al., *Control of allograft rejection in mice by applying a novel neuropeptide, cortistatin.* Adv Ther, 2008. **25**(12): p. 1331-41.
339. You, L., F.E. Kruse, and H.E. Volcker, *Neurotrophic factors in the human cornea.* Invest Ophthalmol Vis Sci, 2000. **41**(3): p. 692-702.
340. Marriott, I. and K.L. Bost, *Substance P receptor mediated macrophage responses.* Adv Exp Med Biol, 2001. **493**: p. 247-54.
341. Kincy-Cain, T. and K.L. Bost, *Substance P-induced IL-12 production by murine macrophages.* J Immunol, 1997. **158**(5): p. 2334-9.
342. Ma, D., et al., *Immunologic phenotype of hosts orally immunized with corneal alloantigens.* Invest Ophthalmol Vis Sci, 1998. **39**(5): p. 744-53.
343. Ma, D., J. Mellon, and J.Y. Niederkorn, *Oral immunisation as a strategy for enhancing corneal allograft survival.* Br J Ophthalmol, 1997. **81**(9): p. 778-84.
344. Ma, D., J. Mellon, and J.Y. Niederkorn, *Conditions affecting enhanced corneal allograft survival by oral immunization.* Invest Ophthalmol Vis Sci, 1998. **39**(10): p. 1835-46.
345. Holzer, P., *Implications of tachykinins and calcitonin gene-related peptide in inflammatory bowel disease.* Digestion, 1998. **59**(4): p. 269-83.
346. Seybold, V.S., et al., *Plasticity of calcitonin gene related peptide neurotransmission in the spinal cord during peripheral inflammation.* Can J Physiol Pharmacol, 1995. **73**(7): p. 1007-14.
347. Scott, D.T., F.Y. Lam, and W.R. Ferrell, *Acute joint inflammation--mechanisms and mediators.* Gen Pharmacol, 1994. **25**(7): p. 1285-96.
348. Pan, X.Q., et al., *Experimental colitis triggers the release of substance P and calcitonin gene-related peptide in the urinary bladder via TRPV1 signaling pathways.* Exp Neurol, 2010. **225**(2): p. 262-73.
349. Bautista, D.M., M. Pellegrino, and M. Tsunozaki, *TRPA1: A gatekeeper for inflammation.* Annu Rev Physiol, 2013. **75**: p. 181-200.
350. Tang, H.B. and Y. Nakata, *The activation of transient receptor potential vanilloid receptor subtype 1 by capsaicin without extracellular Ca2+ is involved in the mechanism of distinct*

substance P release in cultured rat dorsal root ganglion neurons. Naunyn Schmiedebergs Arch Pharmacol, 2008. **377**(4-6): p. 325-32.
351. Kochetkova, I., et al., *IL-35 stimulation of CD39+ regulatory T cells confers protection against collagen II-induced arthritis via the production of IL-10.* J Immunol, 2010. **184**(12): p. 7144-53.
352. Pillai, M.R., et al., *The plasticity of regulatory T cell function.* J Immunol, 2011. **187**(10): p. 4987-97.