Immunoregulatory strategies in corneal transplantation and dry eye disease

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I, William Foulsham, confirm that the work presented in this thesis is my own. Where work has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Despite substantial advances in our understanding of the cellular and molecular factors that regulate immune homeostasis at the ocular surface and anterior segment, important questions remain. In this thesis I present investigations of two phases of the adaptive immune response – the antigen recognition phase, and the final memory phase. In my experiments I employ murine models of corneal transplantation and dry eye disease. Specifically, I investigate how the activation of antigen-presenting cells is modulated by purinergic signaling and by mesenchymal stem cells in a model of corneal transplantation. Furthermore, I investigate how IL-15 signaling regulates the memory T helper 17 cell pool in age-related dry eye disease.

In addition to providing useful models to investigate immunoregulation in the eye, studies of corneal transplantation and dry eye disease are of high translational potential. Corneal transplantation is the most common form of tissue grafting performed worldwide. Despite high success rates in low-risk recipients, failure rates exceed 50% in patients with a history of graft rejection, or with vascularized and inflamed host beds. Dry eye disease is an extremely common chronic condition of the ocular surface, with prevalence estimated to be more than 10% for ages greater than 50 years. Dry eye disease significantly impairs quality of life and is associated with a substantial socioeconomic burden. Accordingly, there is an unmet clinical need for novel therapeutic strategies to improve corneal allograft survival and treat dry eye disease.

The data presented in this thesis indicate that both the purinergic receptor antagonist oxidized adenosine triphosphate and mesenchymal stem cell-derived hepatocyte growth factor promote corneal allograft survival by regulating antigen-presenting cell maturation. In addition, the data presented indicate that targeting interleukin-15 signaling is an effective strategy to deplete the memory T helper 17 pool in aged mice and reduce the severity of age-related dry eye disease.

Impact statement

An estimated ten million people worldwide are blind in both eyes due to corneal disease.[1] Millions more have severe visual impairment secondary to corneal disease. The aetiologies of corneal opacification are diverse and include infections (such as trachoma, onchocerciasis and leprosy), as well as ocular trauma and corneal ulceration. The definitive treatment for corneal blindness is the transplantation of human corneal tissue. However, there is a critical shortage of donor corneal tissue worldwide. Indeed, more than half of the global population does not have access to corneal grafts.[2] Following transplantation (particularly in inflamed eyes and in repeat grafts) a substantial proportion of grafts are rejected by the host's immune system. In order to address the global shortage of donor corneal tissue, numerous attempts have been made (and continue to be made) to manufacture and employ corneal prosthetics.[3,4] In order to reduce the morbidity associated with corneal inflammation, to constrain the potentially blinding inflammation that ultimately necessitates corneal grafting, to increase the success rates of corneal transplantation from human donors and to promote immune tolerance of corneal prosthetics, the development of approaches to regulate the inflammatory response at the cornea is a research priority.

The experiments presented in this thesis employ a murine model of corneal transplantation and a murine model of desiccation-induced dry eye disease in order to study corneal inflammation. Specifically, these experiments are designed to explore the role of the purinergic system (an extracellular signaling system) and two cell types – mesenchymal stromal cells and memory T helper 17 cells – in modulating the inflammatory response. Inside academia, it is anticipated that the publication of the work contained in this thesis will promote future scholarship. Outside academia, aspects of this work have significant translational potential. For example, the first clinical trial of mesenchymal stromal cell-based therapy in corneal transplantation has recently been approved, yet the mechanisms by which mesenchymal stromal cells promote survival of corneal grafts have not been fully deciphered. The work presented in this thesis demonstrates that mesenchymal stromal cell-derived hepatocyte growth factor plays a key role in this process. It is anticipated that unravelling these

mechanisms will be of value to future researchers, but also in the development of novel therapies which may be brought to the clinic.

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Abbreviations

APC	Antigen-presenting cells
ATP	Adenosine triphosphate
CALT	Conjunctiva-associated lymphoid tissue
CD	Cluster of differentiation
CFS	Corneal fluorescein staining
CGRP	Calcitonin gene-related peptide
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DCs	Dendritic cells
DED	Dry eye disease
DLNs	Draining lymph nodes
ELISA	Enzyme-linked immunosorbent assay
FMO	Fluorescence minus one
Foxp3	Forkhead Box P3
FSC	Forward scatter
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GITR	Glucocorticoid-induced tumor necrosis factor receptor
GVHD	Graft-versus-host disease
HGF	Hepatocyte growth factor
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL-1β	Interleukin 1 beta
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked
iTregs	Induced Tregs
mAb	Monoclonal antibody
MHC II	Major histocompatibility complex II
MMPs	Matrix metalloproteinases
MSC	Mesenchymal stem cell
mTh17	Memory T helper 17 cells
NK cells	Natural killer cells
NK1R	Neurokinin 1 receptor
NPY	Neuropeptide Y
oATP	Oxidized adenosine triphosphate
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PD-L1	Programmed death-ligand 1
PEDF	Pigment epithelium-derived factor
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptor
pTregs	Peripherally-generated Tregs
RORγt	RAR-related orphan receptor gamma
SD	Standard deviation
SEM	Standard error of the mean
siRNA	Small interfering ribonucleic acid
SP	Substance P
SSC	Side scatter
TGF-β1	Transforming growth factor beta 1
Th1	T helper 1 cells
Th17	T helper 17 cell
TNFα	Tumor necrosis factor alpha
Tregs	Regulatory T cells
TSP1	Thrombospondin 1
tTregs	Thymus-generated Tregs
VIP	Vasoactive intestinal peptide

Publications arising from work presented in this thesis

Publications directly arising from this work

The purinergic receptor antagonist oxidized adenosine triphosphate promotes corneal allograft survival without expanding regulatory T cells
Foulsham W, Mittal SK, Nakao T, Coco G, Taketani Y, Chauhan SK, Dana R
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Mesenchymal stem cells modulate corneal alloimmunity via secretion of hepatocyte growth factor
Mittal SK*, **Foulsham W*,** Shukla S, Elbasiony E, Omoto M, Chauhan SK **(*Authors contributed equally)**
Stem Cells Translational Medicine 2019; [Epub ahead of print]

An enlarged pool of memory Th17 cells predisposed aged mice to severe exacerbations of dry eye disease
Foulsham W, Mittal SK, Chen Y, Taketani Y, Nakao T, Hong J, Chauhan SK, Dana R
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Additional original research articles published during PhD

Mast Cells Initiate the Recruitment of Neutrophils Following Ocular Surface Injury
Sahu SK, Mittal SK, **Foulsham W**, Li M, Sangwan VS, Chauhan SK.
Investigative Ophthalmology & Visual Science 2018; 59(5): 1732

Mesenchymal Stromal Cells Inhibit Neutrophil Effector Functions in a Murine Model of Ocular Inflammation
Mittal SK, Mashaghi A, Amouzegar A, Li M, **Foulsham W**, Sahu SK, Chauhan SK
Investigative Ophthalmology & Visual Science 2018; 59(3): 1191

Pathological conversion of regulatory T cells is associated with loss of allotolerance
Hua J, Inomata T, Chen Y, **Foulsham W**, Stevenson W, Shiang T, Bluestone JA, Dana R
Scientific Reports 2018; 8(1): 7059

The immunoregulatory role of corneal epithelium-derived thrombospondin-1 in dry eye disease
Tan X, Chen Y, **Foulsham W**, Amouzegar A, Inomata T, Liu Y, Chauhan SK, Dana R
The Ocular Surface 2018; 16(4): 470–7

Mast cells contribute to the induction of ocular mucosal alloimmunity
Li M, Mittal SK, **Foulsham W**, Amouzegar A, Sahu SK, Chauhan SK
American Journal of Transplantation 2019; 19(3): 662-673

Therapeutic efficacy of different routes of mesenchymal stem cell administration in corneal injury
Shukla S, Mittal SK, **Foulsham W**, Elbasiony E, Singhania D, Sahu SK, Chauhan SK
The Ocular Surface 2019; [Epub ahead of print]

Age-related changes to human tear composition
Micera A, Di Zazzo A, Esposito G, Long R, **Foulsham W**, Sacco R, Sgrulletta R, Bonini S.
Investigative Ophthalmology & Visual Science 2018; 59(5): 2024-2031

InflammAging at Ocular Surface: Clinical and Biomolecular Analyses in Healthy Volunteers
Di Zazzo A, Micera A, Coassin M, Varacalli G, **Foulsham W**, De Piano M, Bonini S.
Investigative Ophthalmology & Visual Science 2019; 60(5): 1769-1775

Regulatory T cells promote corneal endothelial cell survival via IL-10
Coco G, **Foulsham W**, Nakao T, Amouzegar A, Taketani Y, Mittal SK, Chauhan SK, Dana R
American Journal of Transplantation 2019; [accepted for publication]

Reviews and book chapters published during PhD

The function of regulatory T cells at the ocular surface
Foulsham W, Marmalidou A, Amouzegar A, Coco G, Chen Y, Dana R
The Ocular Surface 2017; 15(4): 652–9

When Clarity Is Crucial: Regulating Ocular Surface Immunity
Foulsham W, Coco G, Amouzegar A, Chauhan SK, Dana R
Trends in Immunology 2018; 39(4): 288–301

Thrombospondin-1 in ocular surface health and disease
Foulsham W, Dohlman TH, Mittal SK, Taketani Y, Singh RB, Masli S, Dana R
The Ocular Surface 2019; 17(3): 374-383

Therapeutic approaches for induction of tolerance and immune quiescence in corneal allotransplantation
Tahvildari M*, Amouzegar A*, **Foulsham W*,** Dana R (***Authors contributed equally**)
Cellular and Molecular Life Sciences 2018; 75(9): 1509–20

The therapeutic application of mesenchymal stem cells at the ocular surface.
Sahu A, **Foulsham W**, Amouzegar A, Mittal SK, Chauhan SK
The Ocular Surface 2019; 17(2): 198-207

Pathophysiology of Corneal Graft Rejection
Perez VL, **Foulsham W**, Peterson K, Dana R
In: Colby K, Dana R, eds. Foundations of Cornea. New York, NY: Springer. [In Press]

CHAPTER 1. General introduction

1.1. Focused overview of immune system

The immune system describes the collection of tissues, cells and molecules that protect the body against infections. Yet the study of the immune system and its responses – immunology – is critically relevant to good health *beyond its role in fighting infections.* Immune rejection is the primary reason for failure of organ transplantation, the definitive treatment strategy for several end-stage diseases. Failure of the immune system to accurately discriminate between 'self' and 'non-self' can result in life-threatening autoimmune diseases. Furthermore, the immune system is intricately involved in the pathogenesis of neoplastic disease, and immunotherapy is one of the most successful and promising therapeutic modalities for the treatment of human malignancies. The immune system is understood as being comprised of two types of host defense mechanisms: innate immunity (early nonspecific defense against infection) and adaptive immunity (targeted response to a particular pathogen).

1.1.1. Innate immunity

Recognized as the evolutionarily older portion of the immune system, innate immunity comprises both the physical barriers that prevent microbial entry, as well as cellular and molecular mediators that kill or remove microbes that are successful in entering host tissues.[5] Epithelial physical barriers contain specialized intraepithelial immune cells that combat invading microbes, and other biochemical defenses of epithelia include low pH, the secretion of microbiocidal molecules and the production of mucus.[6] In the case of microbes successfully penetrating these physical barriers, they are attacked by other components of the innate immune system including phagocytes, natural killer cells, the complement system and other antibacterial plasma proteins.[6] These mediators of innate immunity recognize structures that are shared by groups of microbes, such as pathogen-associated molecular pattern molecules.[7]

1.1.1.1. Epithelium

Epithelial cells produce enzymes, permeabilizing peptides, protease inhibitors and other binding proteins that neutralize or kill invading microorganisms following activation of pattern-recognition receptors.[8] The T lymphocytes that are contained within epithelia are termed intraepithelial lymphocytes, and include T cells that have receptors composed of α and β chains (as expressed on the most T lymphocytes) as well as T cells with receptors composed of γ and δ chains.[9] Evidence suggests that intraepithelial lymphocytes serve as sentinels against microbial invasion, communicating with both epithelial cells and immune cells that reside outside the epithelium.[10] Epithelial cells play a critical role in the initiation and modulation of adaptive immunity, serving to trigger the maturation of dendritic cells (DCs), as well as the activation and differentiation of B and T lymphocytes.[8] Moreover, through their expression of immunoregulatory molecules such as PD-L1, epithelial cells can function to restrain the adaptive immune response.[8]

1.1.1.2. Goblet cells

Despite enormous functional variations between the mucosal epithelia in the respiratory tract, gastrointestinal tract, genitourinary system and ocular surface, goblet cells are one of the features shared by all these interfaces with the external environment. As specialized secretory epithelial cells, goblet cells release both gel-forming mucins and immunoregulatory molecules.[11] Mucins are categorized as either secreted or membrane-associated, and over 20 of these high molecular weight glycoproteins have been identified to date.[12] Pathogens are trapped in mucus, and are subsequently cleared by physical mechanisms; peristalsis in the gut, mucociliary clearance in the respiratory tract and blinking at the ocular surface.[11]

1.1.1.3. Phagocytes: neutrophils and monocytes

Neutrophils are known as the 'foot soldiers' of the innate immune response. The most abundant leukocytes in human blood, the production of these circulating phagocytes by the bone marrow is promoted by colony-stimulating factors that are secreted by cells in response to infection.[13] Neutrophils are effective in engulfing and destroying microbes in the blood and extravascular tissues, although they have a short lifespan in the tissues, typically dying after only a few hours.[13] Less numerous than neutrophils, monocytes can survive for long periods of time in extravascular tissues, where they differentiate into macrophages.[14] Neutrophils and monocytes migrate to the site of infection or tissue injury via the blood and are guided along chemokine gradients.[14,15]

Upon reaching their destination, the interaction of selectins, integrins and chemokines with their ligands mediate the tethering, rolling and migration through the activated endothelium, respectively.[13] Having recognized microbes by pattern recognition receptors such as Toll-like receptors, neutrophils and monocytes engulf microbes and destroy them via reactive oxygen species, nitric oxide and lysosomal proteases.[14] These enzymes have the potential to injure host tissues if they are released into the extracellular space, and thus phagocytes are capable of considerable collateral damage during the inflammatory response.[13] It is important to note that macrophages perform a diverse amalgam of functions at the intersection of the innate and adaptive immune responses; they contribute to the induction of the adaptive immunity in the lymph nodes, secrete cytokines and growth factors, and act as effector cells of cell-mediated immunity.[16]

1.1.1.4. Mast cells

Mast cells are potent effector cells of the innate immune system that are found in high numbers at mucosal surfaces.[17] Expressing high-affinity Fc receptors for IgE, the cross-linking of IgE bound to these receptors results in mast cell degranulation with the

release of copious pre-formed chemical mediators including histamine, leukotrienes and cytokines.[18] It is this phenomenon that leads to type 1 (i.e. immediate) hypersensitivity reactions such as asthma. In addition to their well-recognized role in allergy, there is an growing body of evidence that mast cells contribute to the immune response *independently* of IgE.[19] Furthermore, published reports indicate that in addition to acting as proinflammatory effector cells, under certain circumstances mast cells may in fact regulate the immune response.[20]

1.1.1.5. Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent stromal cells capable of both self-renewal and differentiation into multiple cell types including osteocytes, chrondrocytes, adipocytes and myocytes.[21] Yet in addition to their capacity to participate in tissue repair by differentiation, MSCs have also been shown to have considerable immunoregulatory capacity.[21] Moreover, current evidence indicates that the immunomodulatory effects of MSCs on particular immune cells are not fixed, but rather are contingent upon their local microenvironment.[21]

1.1.1.6. Dendritic cells

Dendritic cells (DCs) are a major component of both innate and adaptive immunity, serving as a link between the two through their antigen-presenting function.[22] Located in high frequencies in tissues that interface with the external environment, DCs participate in immune surveillance, capturing information about the outside world before migrating to lymphoid tissue to initiate either protective pro-inflammatory or tolerogenic immune responses.[23] Prior to migrating to lymphoid tissues, DCs amplify the innate immune response by producing cytokines that recruit and activate other leukocytes.[24]

1.1.2. Adaptive immunity

Despite innate immunity being a powerful early detection system, many microbes have evolved to evade detection or killing by the innate immune system. It is the adaptive immune system that is responsible for protecting against these infectious agents. The cells that orchestrate the adaptive immune response are lymphocytes, and these cells are activated by their recognition of substances produced by microbes known as antigens.[25] There are two types of adaptive immunity: humoral immunity (involving B lymphocytes) and cell-mediated immunity (involving T lymphocytes). Humoral immunity protects against extracellular microbes, in contrast with cell-mediated immunity that protects against intracellular microbes. In humoral immunity, antibodies are produced by B lymphocytes that neutralize microbes and toxins present in the blood and at mucosal surfaces. In cellular immunity, a portion of T lymphocytes (T helper cells) trigger the destruction of microbes that have been phagocytosed by macrophages, while other T lymphocytes (cytotoxic T cells) directly kill cells infected with intracellular microbes.[25]

The adaptive immune response is characterized by both diversity and antigen specificity.[25] Diversity permits the immune system to mount immune responses following exposure to a large variety of antigens. Specificity ensures that different antigens provoke targeted immune responses. Both diversity and specificity result from the rearrangement of gene segments during development, to produce genes that encode highly specific antigen receptors on lymphocytes.[25] The repertoire of different antigen receptors resulting from this process is enormously diverse, and enables the immune system to respond to a vast array of pathogenic components. Immunologic memory is the phenomenon by which the immune system develops larger and more effective responses following recurrent exposure to the same antigen, and is another defining feature of adaptive immunity. Primary exposure to an antigen results in the generation of populations of long-lived memory T and B cells. Subsequent exposure to

the same antigen results in a secondary immune response, which is both faster and amplified relative to the primary immune response.[25]

1.1.2.1. T cell differentiation and activation

Lymphoid stem cells in the bone marrow are the common source of all lymphocytes, but in contrast to B cells that complete their maturation process in the bone marrow, T lymphocytes mature in the thymus. The cell surface proteins that phenotypically distinguish mature T cells include CD3 (T cell co-receptor), with CD4 and CD8 being expressed by helper and cytotoxic T cells respectively.[26] Having differentiated in the bone marrow, and passed through the positive and negative mechanisms of central selection in the thymus, naïve T cells circulate continuously through peripheral lymphoid organs checking for foreign peptide antigens. The purpose of these naïve T cells is antigen recognition, not the elimination of microbes. In order to defend against pathogens, naïve T cells have to undergo clonal expansion and further differentiation into effector cells.[27] Naïve T cells encounter foreign protein antigens in the peripheral lymphoid organs, where antigens are associated with major histocompatibility complex (MHC) molecules expressed by antigen-presenting cells (APCs). Typically, these APCs are DCs, which are potent activators of naïve T cells due to their high expression of MHC molecules and costimulatory molecules (including CD80 and CD86).[28] In very general terms, effector $CD4^+$ T cells are involved in the activation of macrophages and B cells, while effector $CD8^+$ T cells directly lyse pathogenic cells and also contribute toward macrophage activation.

1.1.2.2. Th1 cells

$CD4^+$ helper T cells differentiate into effector subsets that perform discrete functions and are associated with specific cytokines, cytokine receptors and adhesion molecules.[27] Type 1 helper T (Th1) cells induce the phagocytosis and destruction of microbes, particularly by macrophages.[29] Th1 cells produce numerous cytokines

including interleukin-2 (IL-2) and tumor necrosis factor α (TNFα), but the most critical cytokine produced is interferon γ (IFNγ). IFNγ is a highly effective inducer of antimicrobial and antitumor mechanisms in macrophages, and also upregulates their antigen-presenting functions.[30] By increasing surface expression of FcγRI on mononuclear phagocytes, IFNγ also promotes the elimination of microbes via antibody-dependent, cell-mediated cytotoxicity.[30] Furthermore, by upregulating the expression of complement receptors on mononuclear phagocytes and increasing complement secretion, IFNγ augments complement-mediated phagocytosis.[30]

1.1.2.3. Th2 cells

Th2 cells orchestrate protective immune responses against helminthic parasites by upregulating the production and activity of eosinophils. The cytokine repertoire of Th2 cells includes IL-4, IL-5, IL-9 and IL-13.[31] IL-4 increases the synthesis of IgE antibodies, while IL-5 activates eosinophils. The cytokines secreted by Th2 cells also promote secretion of mucus by goblet cells (thereby augmenting barrier immunity), predispose towards mastocytosis (the abnormal accumulation of mast cells in tissues) and polarize macrophages toward the M2 phenotype (associated with immunoregulatory and tissue repair functions).[31] Th2 cells are also renowned for their role in the inappropriate immune responses underling chronic inflammatory diseases such as asthma and allergy.

1.1.2.4. Th17 cells

The recognition and characterization of Th17 cells as a distinct subset of T helper cells occurred relatively recently. Th17 cells secrete IL-17A, IL-17F and IL-22, and while their putative primary function is the clearance of pathogens resistant to Th1 and Th2 responses, Th17 cells have been implicated in the pathogenesis of a wide range of autoimmune conditions including inflammatory bowel disease, rheumatoid arthritis and multiple sclerosis.[32] Indeed, as a result of the wide distribution of IL-17 and IL-22

receptors, Th17 cells have the capacity to induce extensive tissue inflammation. Th17 cells express the master transcriptional regulator retinoic acid-related orphan receptor γ thymus (RORγt) and differentiation from naïve T cells is induced by IL-6 and transforming growth factor-β (TGF-β). Compared to Th1 and Th2 cells, Th17 cells have demonstrated high levels of context-dependent plasticity and instability.[33] There is evidence that this late plasticity predisposes toward the development of autoimmunity, and yet is also involved in microbial defense and the antitumor function of Th17 cells.[33] Relative to Th1 polarized cells, Th17 cells are associated with an increased capacity for self-renewal and decreased senescence.[33]

1.1.2.5. Regulatory T cells

Despite first being proposed almost half a century ago, little progress was made in our understanding of tolerance-promoting thymic-derived lymphocytes until Sakaguchi's identification of CD25 as a phenotypic marker for $CD4^+$ regulatory T cells in 1995.[34] Following this, Forkhead box protein 3 (Foxp3) was demonstrated to be the transcription factor defining this $CD4^+$ T cell subset in both animals and humans.[35–38] $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) are now recognized as essential for the maintenance of peripheral tolerance, constituting 5-10% of peripheral $CD4^+$ T cells and serving to protect against autoimmune disease and chronic inflammatory conditions.[39] Indeed, mutations of the human gene FOXP3 have been shown to result in the fatal disorder immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX).[40] Yet, in addition to protecting against autoimmunity, Tregs also restrict beneficial immune responses by suppressing antitumor immunity and decreasing responses to vaccines.[39] The majority of $Foxp3^+$ Tregs are thymus-derived regulatory T cells (tTregs), with smaller frequencies of peripherally derived regulatory T cells (pTregs).[41]

Tregs exert their immunosuppressive activity by a variety of mechanisms (Figure 1. 1) (i) cytolysis of effector T cell and antigen-presenting cells via either granzyme-B and perforin-mediated mechanisms or induction of apoptosis via TRAIL-DR signaling, (ii) the release of soluble immunoregulatory factors such as IL-10, TGF-β and IL-35, (iii) modulation of dendritic cell maturation via constitutively expressed cell surface molecules such as cytotoxic T-lymphocyte antigen 4 (CTLA-4), and (iv) by competing with effector T cells for key metabolites such as IL-2, leading to cytokine deprivation-mediated apoptosis.[39,42]

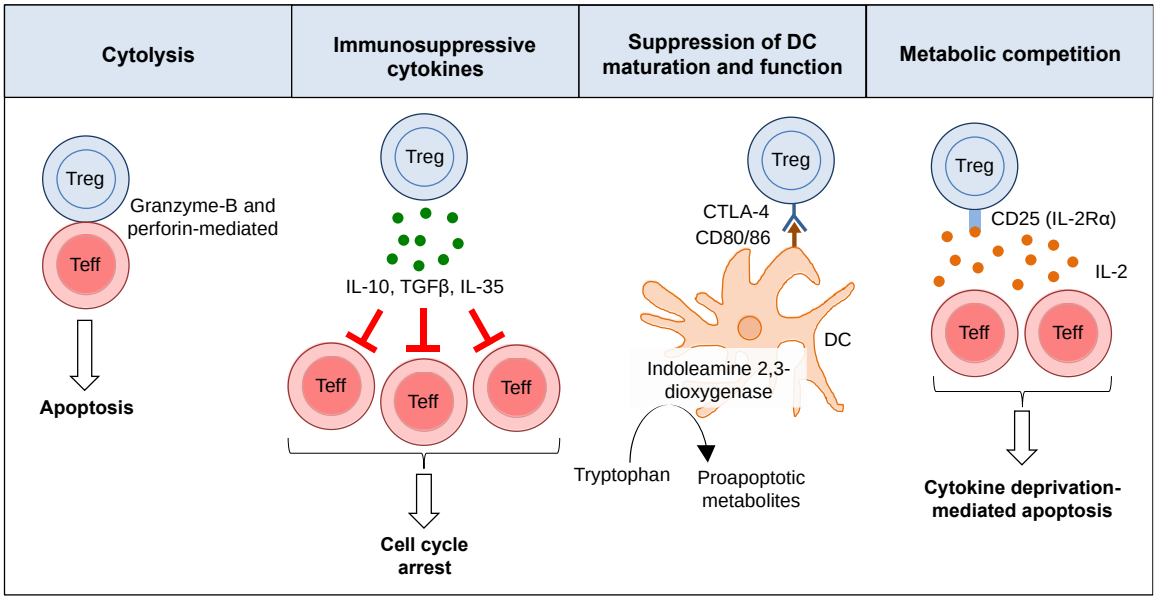

Figure 1. 1 Treg immunoregulatory mechanisms
Tregs use four main mechanisms to modulate inflammation: (i) cytolysis of effector T cells, (ii) the release of soluble immunosuppressive factors, (iii) control of dendritic cell (DC) function and (iv) metabolic competition for IL-2. From Foulsham et al. 2018.

The developmental pathways of Th17 cells and $CD4^+CD25^+Foxp3^+$ Tregs are reciprocally interconnected.[43] Tregs and Th17 require TGF-β for their development, which is necessary for inducing both Foxp3 and RORγt.[43] In a quiescent immune environment, TGF-β-induced Foxp3 inhibits RORγt function and thereby limits Th17 differentiation.[44] The presence of the pro-inflammatory cytokines IL-6 or IL-21 reduces the expression of TGF-β induced Foxp3, resulting in increased expression of RORγt, and the Th17/Treg balance tilts in favor of Th17 generation.[43] As with Th17 cells,

differentiated Tregs exhibit a high grade of plasticity.[45] Indeed, the presence of TGF-β and IL-6 can reprogram Tregs into Th17 cells.[46] The balance between Th17 and Treg development, and the functional stability of these $CD4^+$ T cell subsets, have been shown to be critically important factors in both immune tolerance of allografts and the pathogenesis of autoimmunity.[45,47]

1.2. Ocular surface anatomy

The ocular surface is comprised of the cornea, conjunctivae and tear film.[48] The cornea contributes approximately two thirds of the eye's refractive power, and thus corneal smoothness and optical clarity is essential for visual function.[49] As the most exposed mucous membrane of the body, the maintenance of these optical properties requires protection against inflammatory and fibrotic processes. A nonkeratinized stratified squamous epithelium covers the cornea, limbus and conjunctiva and shares a similar structure to the mucosa of the esophagus and oral cavity.[50] Here, a concise overview of the anatomical features of the ocular surface is provided.

1.2.1. Cornea

In addition to providing the majority of the eye's refractive power, the cornea must be mechanically extremely tough to protect the inner contents of the eye.[49] The normal corneal is avascular, with the majority of metabolic substrates being provided either by the tear film (e.g. oxygen) or the aqueous humor (e.g. glucose).[51] The cornea is one of the most densely innervated and sensitive tissues in the human body; and these nerves play a role in blinking (i.e. physical protection), inducing reflex tear production and stimulating the release of trophic factors.[52] The average thickness of the central cornea is 540 μm, with thickness increasing toward the corneal periphery.[53] On average, the vertical diameter is 11.5 mm and horizontal diameter 12 mm.[53]

Figure redacted for copyright reasons

Figure 1. 2 Structure of the cornea
From Bowling 2016.

The human cornea is comprised of five layers – the corneal epithelium, a fibrous meshwork called Bowman's layer, the stroma (a collagen-rich central layer that represents 90% of corneal thickness), Descemet's membrane and the corneal endothelium (Figure 1. 2).[49] The epithelium is a nonkeratinized, stratified squamous epithelium that is approximately 50–60 μm thick and consists of five or six layers of cells. Hemidesmosomes anchor a single layer of columnar basal cells to the underlying basement membrane.[53] Desmosomes hold adjacent cells together. Microvillae and microplicae (ridges) cover the anterior surface of the corneal epithelium with an overlying glycocalyx coat which plays a key role in maintaining the precorneal tear film.[54] The corneoscleral limbus contains stem cells, the mitotic activity of which produces new cells that migrate centripetally and superficially to displace existing cells. It is noteworthy that deficiency or disruption of the limbal stem cell niche can result in chronic epithelial defects and corneal blindness.[55] Beneath the corneal epithelium, there is an acellular superficial layer of the stroma of 8 - 12 μm thickness composed of randomly arranged collagen fibrils called Bowman's layer.[56]

The corneal stroma comprises 90% of the thickness of the cornea, and consists predominantly of type I collagen fibers, although some type III, V and VI fibers are also present.[54] These fibers form lamellae that are organized parallel to the corneal surface, and have keratocytes (modified fibroblasts) positioned between them.

Maintenance of the stromal architecture is essential for corneal clarity.[49] In the normal eye, the stroma does not contain either blood or lymphatic vessels.

Descemet's membrane is an 8 - 12 μm discrete layer of latticed collagen fibrils that is positioned in between the corneal stroma and the endothelium.[53] Descemet's membrane is comprised of two parts; the anterior banded zone that is deposited *in utero*, and the posterior non-banded zone that is continuously laid down by the corneal endothelium. Indeed, Descemet's membrane serves as a modified basement membrane for the endothelium, which is a simple squamous epithelium on the posterior surface of the cornea.[54] In young adults, the density of corneal endothelial cells is approximately 3000 cells/mm^2, and this density decreases with age.[57] The corneal endothelium plays a vital role in maintaining corneal hydration, and in cases where cell density drops below 800 cells/mm^2, corneal edema and swelling occur leading to impairment of vision.

1.2.2. Conjunctiva

The conjunctiva is a mucous membrane that lines the inner aspect of the eyelids and the anterior surface of the globe. The conjunctiva is richly vascular with dense lymphatic networks.[53] It is composed of three parts – the palpebral conjunctiva (running from the lid margins to the posterior tarsal plates), the forniceal conjunctiva (loose middle connecting segment) and the bulbar conjunctiva (covering the anterior sclera from the fornix to the corneoscleral limbus). There are 3-5 layers of cells that constitute the conjunctival epithelium, which is non-keratinized.[58] Mucin-producing goblet cells are scattered throughout the conjunctival epithelium. The accessory lacrimal glands of Krause and Wolfring are situated in the conjunctival stroma, and represent approximately 10% of the total lacrimal secretory mass.[59] Intraepithelial lymphocytes, subepithelial follicles, lymphatics and blood vessels in the conjunctiva

comprise the conjunctiva-associated lymphoid tissue (CALT), which plays a role in protecting the ocular surface by triggering and modulating immune responses.[60]

1.2.3. Tear film

The tear film serves to protect and moisturize the cornea, it provides a smooth optical surface and permits the diffusion of oxygen and other nutrients. The tear film has classically been understood as being composed of three layers – a superficial lipid layer, an aqueous layer, and a mucin layer covering the corneal epithelium (Figure 1. 3).[61] The superficial lipid layer is produced predominantly by the Meibomian glands and functions to prevent evaporation and overspill of the aqueous layer, which in turn provides nutrients, antimicrobial proteins, lubrication and determines osmolarity.[62] The aqueous layer is produced by the main and accessory lacrimal glands. The basal mucin layer is derived from conjunctival goblet cells, and functions to lower the hydrophobicity of the epithelial cells.[54] Interestingly, the aqueous layer was previously considered to occupy >90% of the total tear film depth, but laser inferometry studies by Prydal et al.[63] amongst others suggested that the mucous layer in fact represented a larger proportion. More recently, there has been a shift toward understanding the precorneal tear film as a single dynamic functional unit with different compartments, rather than the three layered model.[62]

Figure redacted for copyright reasons

Figure 1. 3. Layers of tear film

From Bron et al. 2003. Note that this is based on model by Prydal et al. 1992 in which majority of thickness is mucous layer.

1.3. Immune homeostasis at the ocular surface

The ocular surface mucosa has several features in common with the mucus membranes of the respiratory, gastrointestinal and urogenital tracts. Yet the ocular surface has a physiological requirement that is unique among these mucosae – optical clarity. Thus, a successful inflammatory response should not compromise corneal transparency, but rather find a middle path by which microbial insults are eliminated without excessive collateral damage to bystander cells.[50]

Along with the brain, placenta and testes, the eye has long been considered a tissue that enjoys 'immune privilege'.[64] The term was coined by Peter Medawar, who in the 1940s had observed decreased rejection rates of allogeneic skin grafts situated heterotopically in the anterior chamber of the eye.[65] Medawar ascribed the relative immunological restraint in the anterior chamber of the eye to an apparent absence of lymphatic drainage, with the resultant sequestration of antigens. Although what Medawar described (now referred to as 'immunological ignorance') represents an important part of the modern concept of ocular immune privilege, we now understand this phenomenon to encompass a much wider combination of anatomical attributes, physiological processes and immunoregulatory mechanisms that act in concert to constrain the immune response.[50]

1.3.1. Immune regulation by the ocular surface epithelium

The ocular surface epithelium functions to limit inflammation by a variety of surface-expressed and secreted molecules (Table 1. 1).[50] Corneal epithelial cells constitutively express surface-bound immunoregulatory molecules such as programmed death ligand 1 (PDL-1)[66] and Fas ligand (FasL)[67]. Corneal epithelial cells constitutively secrete pigment epithelium-derived factor (PEDF)[68] and endogenous vascular endothelial growth factor traps such as sVEGFR1[69] and VEGFR3[70], which also serve to suppress the inflammatory response. There is evidence that ocular surface

epithelial cells interact with DCs to promote their tolerogenicity via a thrombospondin-1-dependent mechanism.[71] DC function is also regulated by thymic stromal lymphopoietin, which is expressed by corneal epithelial cells.[72]

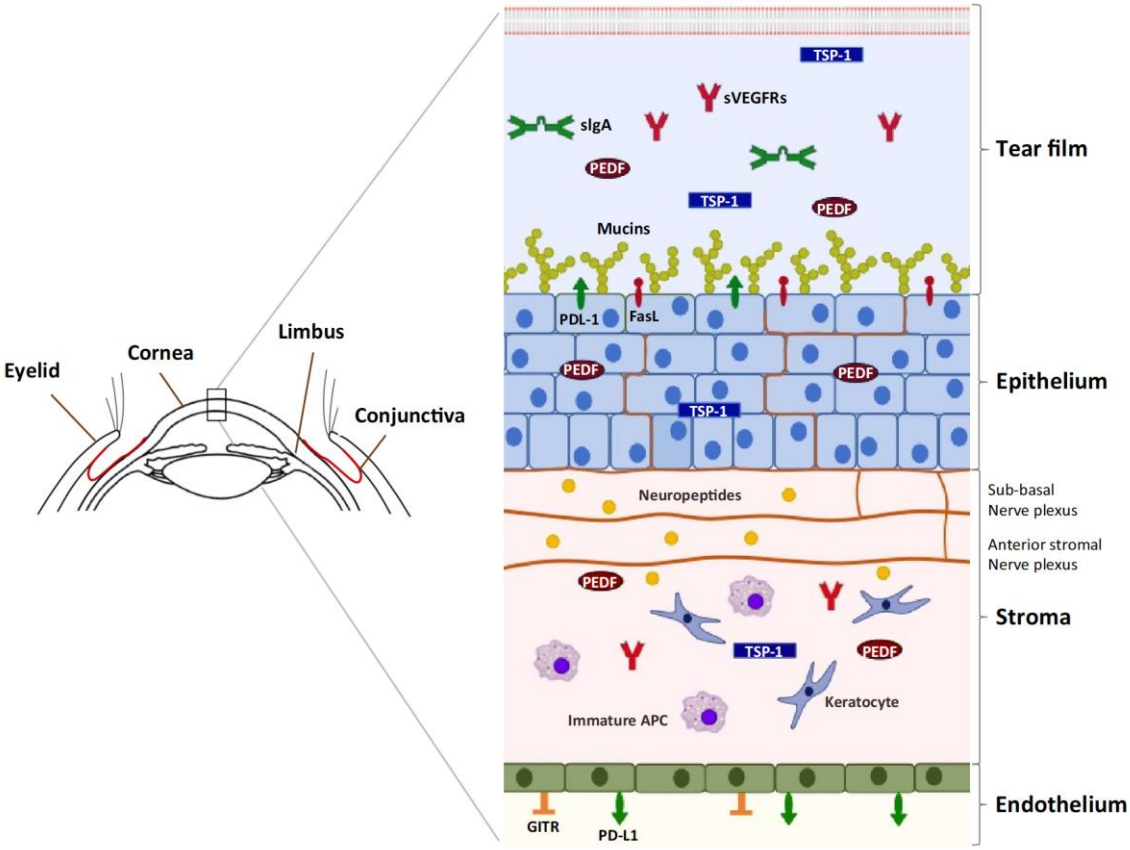

Figure 1. 4 Ocular surface immunoregulatory factors

Immunomodulatory factors expressed by the corneal epithelium include cell-surface molecules (including FasL and PDL-1) and secreted factors (including PEDF and the soluble VEGF decoy receptors). Secretory IgA (sIgA) in tear film serves to facilitate cytolysis and suppress the proliferation of pathogenic organisms. Membrane associated mucins of the glycocalyx function to limit bacterial adherence and facilitate the clearance of debris via the lacrimal puncta. Nerves in the sub-basal and anterior stromal nerve plexuses modulate both innate and adaptive immune responses via neuropeptides including CGRP and VIP. Posterior to the stroma, the corneal endothelium expresses immunomodulatory molecules such as PDL-1 and GITR. From Foulsham et al. 2018.

1.3.2. Immune regulation by the tear film

The tear film operates as a mechanical barrier to pathogenic mediators, as in the process of blinking and tearing antigens are washed away from the ocular surface.[73] Secretory IgA is the primary immunoglobulin in the tear film, which has been shown to play a role in regulating DC activity, as well as the neutralization of bacteria, toxins and viruses.[74] Other antimicrobial molecules found in the tears include lysozyme, lipocalin

and lactoferrin.[73] High molecular weight heavily glycosylated membrane-associated mucins in the glycocalyx help to prevent bacterial adhesion and invasion, and secreted gel-forming mucins function to trap debris for elimination via the lacrimal puncta.[75]

	Molecules	Function
Regulatory cytokines	TGF-β IL-1Ra	TGF-β functions pleiotropically – it inhibits the proliferation of T cells and induces Foxp3$^+$ inducible Tregs, yet in the presence of IL-6 induces pathogenic IL-17-producing Th17 cells.[76] In addition, TGF-β plays a critical role in tissue fibrosis.[77] IL-1Ra is upregulated in ocular surface inflammation, and functions to block the actions of IL-1 and suppress leucocyte/APC activity.[78]
Inhibitory factors	PD-L1 FasL TRAIL TSP-1	PD-L1, FasL and TRAIL promote apoptosis of activated immune cells, limit T cell proliferation and suppress inflammatory cytokine production.[79] TSP-1 activates the immunomodulatory cytokine TGF-β.[71]
Neuropeptides	Substance P α-MSH CGRP VIP	Substance P functions pleiotropically – it stimulates the recruitment of monocytes and neutrophils, inhibits IL-10 production by DCs and promotes Th17 production, yet also induces Tregs.[80,81] α-MSH, CGRP and VIP dampen the recruitment of inflammatory cells, limit T cell proliferation and suppress production of inflammatory cytokines.[82] α-MSH can also promote Treg function.[83]
Hemangiogenic and lymphangiogenic factors	sVEGFR-1 sVEGFR-2 VEGFR-3 PEDF	Hemangiogenesis and lymphangiogenesis are limited by the constitutive expression of VEGF decoy receptors, which are upregulated in ocular surface inflammation.[69,70,84–86] PEDF suppresses VEGF expression, and its expression is decreased in ocular surface inflammation.[68,87]

Adapted from Foulsham et al. 2018.

Table 1. 1 Select immunoregulatory factors at the ocular surface

1.3.3. Ocular surface dendritic cells

The conjunctiva contains the highest density of DCs, and their frequencies decrease from the limbus to the center of the of the cornea.[88] Peripheral tissues contain steady-state DCs that continuously sample and process antigen from the local microenvironment and play a critical role in initiating adaptive immune responses.[89] Membrane nanotubes have been identified on MHCII$^+$ cells in the mouse cornea *in vivo*, and it has been suggested that these may transport antigen from the tear film to

DCs, thus having an analogous function to intraepithelial DCs in the respiratory and intestinal tracts.[89,90]

The main DC subsets have been identified at the ocular surface (Figure 1. 5).[91,92] Langerhans cells have been identified in the peripheral corneal epithelium, and DC-SIGN$^+$ DCs have been identified in the peripheral anterior corneal stroma.[93] This mirrors the pattern of distribution in the skin, where the epidermis contains Langerhans cells and the dermis contains DC-SIGN$^+$ DCs.[93] Although MHCII$^+$ Langerhans cells are found in the peripheral cornea and limbus, these cells are not identified in the central cornea in the uninflamed setting.[94] A population of CD103$^+$ DCs that express Langerin but are not Langerhans cells have been identified in the corneal stroma, further echoing findings from the skin.[95] The anterior corneal stroma (both centrally and peripherally) has been shown to contain MHCII-negative, costimulatory molecule-negative DCs.[96] In response to inflammation, these cells undergo maturation with increased expression of MHCII, CD40, CD80 and CD86.[94,97] CD11b$^+$ DCs have also been observed in the posterior corneal stroma and conjunctiva.[93,98] There is some evidence that in addition to promoting inflammation at the ocular surface, subpopulations of DCs may contribute toward immune tolerance.[48,92] Indeed, in the setting of corneal transplantation, the therapeutic efficacy of tolerogenic DCs has been demonstrated.[99] However, further research is needed to precisely characterize the DC phenotypes that promote immune tolerance at the ocular surface.[50]

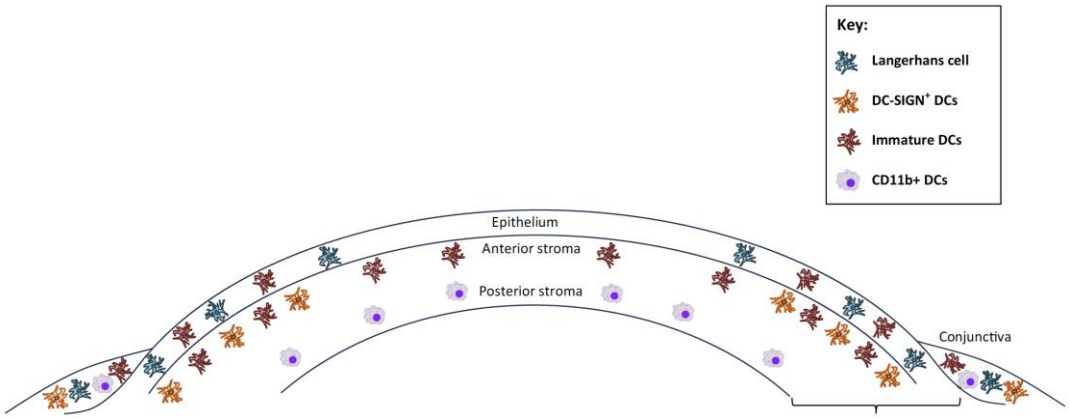

Figure 1. 5 Ocular surface dendritic cells

The highest frequencies of DCs are found in the conjunctiva, and their density decreases from the limbus to the center of the cornea. The peripheral corneal epithelium and conjunctiva contain Langerhans cells. The peripheral anterior stroma and conjunctiva contain DC-SIGN$^+$ DCs. The posterior stroma and conjunctiva contain CD11b$^+$ DCs. Immature DCs are located in the anterior stroma and undergo maturation with increased expression of MHCII and costimulatory molecules (CD80, 86 and CD40) upon exposure to an inflammatory microenvironment. From Foulsham et al. 2018.

1.3.4. Neuropeptides

The cornea is the most densely innervated tissue in the human body, and neuropeptides (short chain polypeptides that act as signaling molecules between neurons) are known to regulate immunity at the ocular surface.[82] Neuropeptides modulate both the innate and adaptive immune responses, and have been shown to have both proinflammatory and immunoregulatory effects. For example, substance P (SP) has been shown to have predominantly proinflammatory activity, and functions to recruit neutrophils and monocytes, increase mast cell degranulation and promote Th17 maturation.[80,81] Intriguing work conducted using a murine model of corneal transplantation indicates that SP secretion is induced *bilaterally* after corneal nerves are severed *unilaterally*, resulting in impaired regulatory T cell function in both eyes and decreased survival of corneal transplants.[100] Studies using mice lacking the receptor for SP (neurokinin-1 receptor, NK1R) have demonstrated reduced corneal nerve density and upregulated desquamation of apical epithelial cells in these mice.[101]

Other neuropeptides such as calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY) and vasoactive intestinal peptide (VIP) have been shown to promote immune

quiescence at the ocular surface.[82] CGRP inhibits the production of IL-2, suppresses the maturation of DCs and inhibits the secretion of inflammatory cytokines by macrophages.[102] NPY has been shown to regulate immune cell trafficking and cytokine secretion; as well as to modulate NK cell activity, T helper cell differentiation and macrophage function.[103,104] VIP induces tolerogenic DCs, inhibits the expression of chemokines and suppresses the release of proinflammatory cytokines.[105] Thus numerous anti-inflammatory neuropeptides work in concert to limit the potency of the immune response at the ocular surface.

1.4. Corneal transplantation

1.4.1. Epidemiology, indications and survival rates

More than 100 years ago, the Austrian ophthalmologist Eduard Zirm reported the world's first human full-thickness corneal transplant that remained clear post-operatively.[106] This event represents a significant milestone in corneal grafting becoming what it is today - the most commonly transplanted tissue worldwide.[107] Indeed, over 150,000 corneal transplantation procedures are conducted globally each year.[2]

Over recent years, two notable trends have been occurring in the field of corneal transplantation. Firstly, the number of lamellar keratoplasty procedures (i.e. partial-thickness grafts) performed has been dramatically increasing[2] and is used in clinical scenarios where full-thickness grafts would previously have been indicated. Likely a result of the reduced allogeneic load, these partial thickness grafts enjoy lower rates of immune rejection than penetrating keratoplasty.[108] Yet despite improvements in lamellar keratoplasty techniques, full-thickness corneal transplants remain the definitive treatment for many end-stage corneal diseases including keratoconus, traumatic corneal scarring and corneal dystrophies.[109] Secondly, with improved eye banking services and distribution networks, the number of full-thickness corneal transplants performed in developing countries is increasing.[2] This trend is likely to continue as efforts to promote cornea donation and delivery improve services for the 53% of the global population without access to corneal transplantation.[2]

Due to the relative quiescence of the immune microenvironment, corneal transplantation enjoys high success rates in patients with non-vascularized and uninflamed host beds receiving their first corneal transplant. Indeed, in this setting five-year survival rates exceed 90% without human leukocyte antigen matching or systemic immunosuppressive drugs.[110] However, for repeat grafts, or when there is

pre-existing vascularization and inflammation of the host bed, rejection rates exceed 50% despite maximal local and systemic immunosuppressive therapy.[109,111]

Immune rejection is the primary cause of graft failure.[112] Graft rejection can occur in the corneal epithelium, stroma or endothelium, or a combination of these.[113] The most common form is endothelial rejection, occurring in 50% of rejection episodes.[114] Endothelial rejection is characterized by a line of keratitic precipitates beginning at the graft-host junction and progressing across the endothelium over time. The pathognomic endothelial rejection line is known as 'Khodadoust's line'.[113] Other ocular signs include conjunctival hyperaemia, graft oedema (due to failing of the endothelial pump function to control corneal hydration) and anterior chamber reactions.[115]

1.4.2. Corneal transplantation: immunobiology of graft rejection

The survival of allogeneic corneal grafts is determined by the balance between pro-inflammatory responses that promote immune rejection, and immunomodulatory mechanisms that promote tolerance.[79] Following transplantation, the upregulated expression of pro-inflammatory cytokines, adhesion molecules, chemokines and pro-angiogenic factors drives infiltration of immune cells into the cornea, and promotes the development of new blood and lymphatic vessels.[79] In this inflammatory milieu, both host and donor APCs undergo maturation with upregulated expression of MHCII and co-stimulatory molecules including CD80 and CD86.[96,116] Mature APCs egress from the cornea and migrate via lymphatic vessels to the draining lymph nodes. In the draining lymph nodes, APCs present alloantigens to naïve T cells (Th0), thereby triggering differentiation and clonal expansion into IFNγ-secreting $CD4^+$ Th1 cells.[79] Regulatory T cells (Tregs) limit the induction of alloimmunity by releasing soluble immunosuppressive factors, by regulating APC stimulatory potential, by metabolic competition and by cytolysis of Th1 cells.[42] Alloreactive Th1 cells egress the lymph nodes and migrate via blood vessels toward the graft, following a chemokine gradient.

At the cornea, the Th1 cells effect a delayed-type hypersensitivity response toward the graft, with the release of pro-inflammatory mediators, thereby driving rejection.[79]

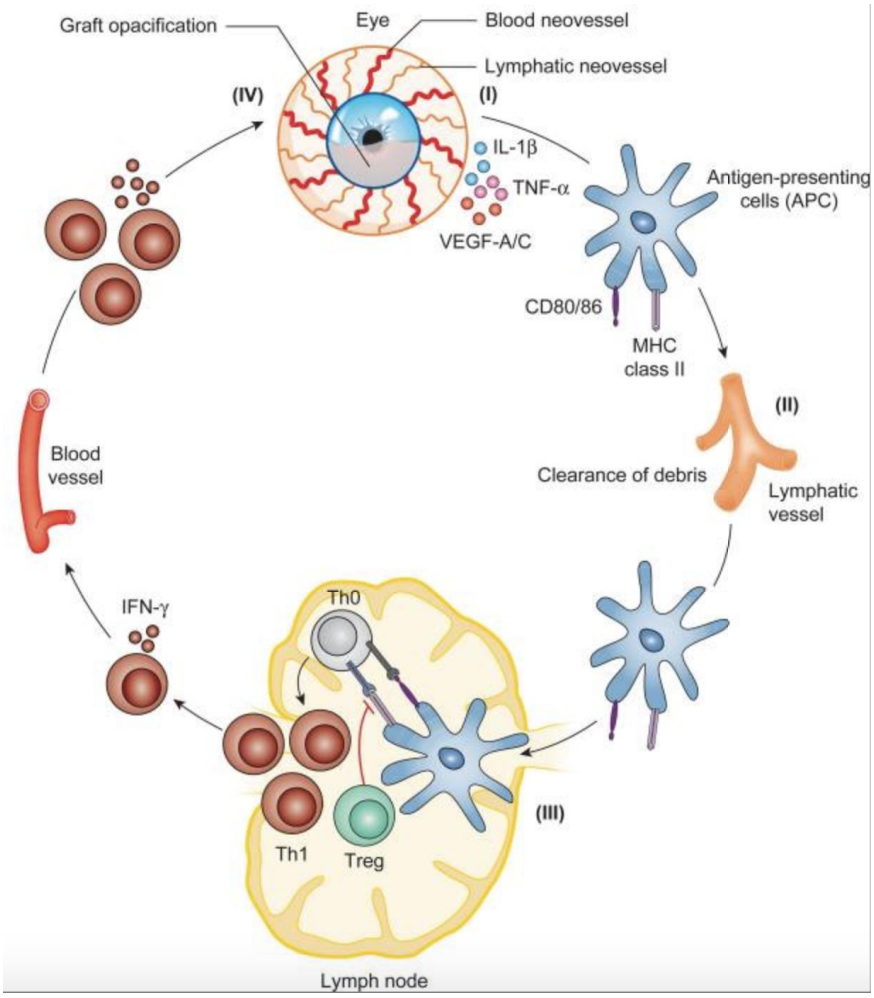

Figure 1. 6 Immunobiology of corneal transplantation

An inflammatory microenvironment at the graft site activates antigen presenting cells (APCs) (**I**), which migrate via lymphatic vessels to the draining lymph nodes (**II**). Here, APCs promote the differentiation of IFNγ-secreting $CD4^+$ Th1 cells, a process that is modulated by regulatory T cells (**III**). Th1 cells migrate via blood vessels to the graft site, where they promote immune rejection (**IV**). From Amouzegar et al. 2016

1.4.2.1 Direct vs. indirect allosensitization

At early timepoints following corneal transplantation, the innate immune response results in increased expression of a diverse array of proinflammatory factors, including cytokines (e.g. IL-1, IL-6, TNFα), chemokines, adhesion molecules and pro-angiogenic factors.[117–121] This inflammatory microenvironment results in the activation of *both* resident donor and infiltrating host APCs, with the increased expression of MHCII and costimulatory molecules including CD80, CD86 and CD40. Notably, DCs and other

myeloid cells that are resident in the centre of the cornea constitutively express negligible levels of MHCII, in contrast with the peripheral cornea which contains DCs that do express MHCII.[122] Yet corneal inflammation (for example in the setting of corneal transplantation) has been shown to induce MHCII expression by the majority of both corneal DCs and epithelial cells.[123]

There are two pathways by which allosensitization occurs. The direct pathway involves 'passenger leukocytes' (i.e. donor-derived APCs) presenting donor antigens to host naïve T cells.[97] The indirect pathway involves recipient APCs from the peripheral cornea presenting donor antigens to host naïve T cells.[97] Host APCs from the limbal vasculature are induced to migrate centripetally toward the corneal graft by adhesion molecules and chemokines, the expression of which are upregulated in an inflamed microenvironment.[116,124] Interestingly, it was previously thought that graft antigens were *exclusively* recognized via the indirect pathway in corneal transplantation.[125] However, subsequent studies identifying populations of resident DCs in the cornea that selectively express MHCII under inflammatory conditions have implicated the direct pathway of allosensitization in corneal graft rejection.[94,96] Moreover, consistent with the observation that MHCII expression by these cells is induced by an inflammatory milieu, there is evidence that the direct pathway plays a proportionally greater role in allosensitization when the host bed is inflamed.[97] Using a murine model of high-risk corneal transplantation in which inflammation and vascularization are induced in the recipient bed by placing stromal sutures two weeks prior to transplantation, Huq and colleagues have shown that the survival of grafts sourced from MHCII knockout mice significantly enhanced survival in high-risk, but not low-risk hosts.[97] These and other data[94,96,97] emphasize the importance of the direct pathway of allosensitization in corneal transplantation, and highlight the relevance of the graft microenvironment to this process.

1.4.2.2 Lymphangiogenesis and haemangiogenesis

Mature APCs migrate from the cornea to the draining lymph nodes via lymphatic vessels. Following differentiation and clonal expansion, graft-attacking Th1 cells migrate from the draining lymph nodes to the graft site. Ocular inflammation upregulates both of these processes by promoting the formation of new lymphatic and blood vessels. The importance of these phenomena in determining graft outcomes is evidenced by the dramatically different survival rates in high-risk (i.e. stromal sutures placed two weeks prior to transplantation) compared to normal-risk murine models of corneal transplantation.[126,127] Furthermore, in a study using the high-risk model, 100% of grafts have been observed to be rejected at 5 weeks post-transplantation, yet when cervical lymphadenectomy was performed prior to transplantation, rejection rates fell to less than 10%.[86] Pharmacological targeting of VEGF pathways in the high-risk murine model of corneal transplantation has also been demonstrated to reduce graft rejection.[85,128,129]

The infiltration of innate immune cells into the inflamed cornea is promoted by Intercellular Adhesion Molecule 1 (ICAM1), C-C Motif Chemokine Ligand 2 (CCL2), CCL20 and inflammatory cytokines, which in turn produce VEGF-C and VEGF-D to stimulate lymphangiogenesis.[121,130–133] During their activation, APCs gain expression of VEGFR-3, which further promotes the homing of APCs along a chemotactic gradient to the draining lymph nodes.[134] Interactions between APC-expressed CCR7 and its ligand CCL21 have also been demonstrated to be important in both mediating APC migration to the draining lymph nodes, and have been shown to regulate integrin-mediated APC transmigration across lymphatic endothelium.[135,136]

1.4.2.3 Effector immune cells in corneal transplantation

There is general consensus that the primary effector cells driving corneal graft rejection are IFNγ-secreting $CD4^+$ Th1 cells.[137,138] High expression of Th-1 associated

cytokines, such as IFNγ and IL-2, have been observed in grafts undergoing rejection.[139,140] However, Th1 cells are by no means the exclusive cell population driving the alloimmune response. Indeed, despite a decreased rate of rejection, 45% of allogeneic corneas grafted onto CD4 knockout recipients have been observed to be rejected at 16 weeks post-operatively.[138] Another study in which graft recipients were treated systemically with anti-CD4 monoclonal antibody demonstrated a rejection rate of 33% despite CD4 depletion.[139] High rejection rates have also been observed when IFNγ knockout mice were used as recipients,[141] further indicating that effector cells other than Th1 are promoting graft rejection.

Th17 cells have been implicated in the early stage of immune rejection of corneal transplants.[142] In this study, deficiency of IL-17 was observed to delay the development of the allogeneic response, but did not influence overall survival.[142] This observation may be related to delayed lymphangiogenesis secondary to IL-17 deficiency.[143] Interestingly, another study from the Niederkorn group has reported that depletion of IL-17 *increased* the incidence of corneal allograft rejection from 50 to 90%.[144] Further investigations have suggested that this phenomena may be attributable to the close interplay between Th17 cells and $CD4^+CD25^+$ Tregs, which play a critical role in promoting immune tolerance at the ocular surface.[42,145]

There is some evidence for the involvement of $CD8^+$ T cells in promoting graft rejection from both murine[125,146] and rat[147–149] studies. In contrast, studies using beta-2 microglobulin knockout mice (deficient in $CD8^+$ T cells) and perforin knockout mice provide evidence for the redundancy of $CD8^+$ T cells in corneal allograft rejection.[150,151] Moreover, experiments determining the antigen specificity, frequency and phenotype of T cells activated via direct and indirect pathways suggest that although alloreactive $CD8^+$ T cells *are activated* via the direct pathway, these cells do not contribute to graft rejection unless they receive additional signalling from APCs in the periphery.[152]

1.4.2.4 Regulatory immune cells in corneal transplantation

After exposure to alloantigens *in vivo,* $CD4^+CD25^+Foxp3^+$ Tregs exhibit alloantigen-specific immunomodulatory activity.[153,154] In corneal transplantation, Treg expression of Foxp3 has been shown to be directly associated with their production of regulatory cytokines (including IL-10 and TGF-β), suppression of effector T cell activation and their capacity to prevent allograft rejection.[155] The soluble regulatory molecules IL-10 and TGF-β are known to play an important part in promoting allograft survival.[156–158] Regulatory T cells can be classified into thymus-derived natural Tregs (tTregs) and peripherally-induced Tregs (pTregs).[41] Using the high-risk allogeneic murine model of corneal transplantation, Inomata and colleagues have demonstrated that an inflammatory microenvironment selectively reduces the frequencies and function of pTregs, but not tTregs.[159] In their study, the investigators observed the impaired suppressive function of Tregs to be associated with reduced expression of the immunomodulatory molecules IL-10, TGF-β and CTLA-4.

The observation that subpopulations of Tregs are predisposed to functional impairment is related to the concept of Treg plasticity. The phenotypic plasticity and functional adaptability of Tregs in inflammatory milieux has been demonstrated in studies of autoimmune disease.[160,161] Based on experiments employing lineage-reporter mice, there is evidence that pTregs are particularly liable to lose expression of Foxp3, and to express the pro-inflammatory cytokines IL-17 or IFN-γ, in response to cues from an inflammatory microenvironment.[162] Indeed, the developmental pathways of Th17 cells and Tregs are closely and reciprocally interconnected, and both cells types exhibit considerable plasticity.[43] Interestingly, there is evidence that IL-17 is critically involved in mediating Treg contact-dependent suppression.[145] In this study, Cunnusamy and colleagues demonstrated that inhibition of IL-17 signalling with anti-IL-17 monoclonal antibody resulted in rejection of 90% of corneal allografts.

Tregs isolated from allograft acceptors, but not rejectors, have been shown to colocalize with APCs in the lymph node paracortex and express high levels of CCR7.[163] Moreover, there is evidence that CCR7 expression upregulates Treg suppressive function, in addition to mediating Treg homing.[163] Augmenting Treg expression of CCR7 by conditioning Tregs *ex vivo* with the CCR7 ligand CCL21 has been shown to enhance their capacity for promoting allograft survival.[163]

In addition to modulating the immune response in the draining lymph nodes, local delivery of Tregs to the graft site has been shown to promote transplant survival.[164] Shao and colleagues injected Tregs subconjunctivally into corneal transplant recipients, demonstrating reduced APC maturation both in the graft and draining lymph nodes, reduced Th1 frequencies in the draining lymph nodes and enhanced expression of the immunomodulatory cytokines IL-10 and TGF-β at the graft site.[164] These observations were associated with increased allograft survival in the Treg-treated mice. Other approaches to Treg immunotherapy in corneal transplantation include the *in vivo* expansion of Tregs using low-dose interleukin-2 (IL-2) treatment.[165] Systemic administration of IL-2 was shown to increase both Treg frequencies and suppressive function, with decreased Th1 frequencies and decreased graft infiltration of $CD45^+$ inflammatory cells observed following transplantation.[165] Clinical assessment of the grafts demonstrated reduced opacity and prolonged survival in the IL-2-treated animals.[165]

Maturation-resistant tolerogenic DCs have also attracted attention as potential candidates to induce tolerance in corneal transplantation.[79] Expressing low levels of MHCII and co-stimulatory molecules, donor-derived tolerogenic DCs have been shown to increase Treg frequencies and promote corneal allograft survival when administered prior to transplantation.[166,167] There is evidence that tolerogenic DCs increase corneal

graft survival by upregulating frequencies of CTLA-4-expressing Tregs, and decreasing frequencies of $CD28^+$ Tregs.[167] Finally, enrichment of tolerogenic APCs in donor corneas prior to transplantation through incubation with IL-10 and TGF-β has been shown to reduce host allosensitization, decrease frequencies graft-attacking Th1 cells and reduce graft infiltration of $CD45^+$ and $CD4^+$ cells, with improved transplant survival observed.[168]

1.5. Mesenchymal stem cells

1.5.1. Immunomodulatory functions of mesenchymal stem cells

Mesenchymal stem cells (MSCs), also known as mesenchymal stromal cells, are found in almost all tissues, are self-renewing and have the capacity to differentiate into specialized cell types.[169] Renowned for their plasticity, it is postulated that MSCs contribute to cellular homeostasis by supplanting dysfunctional or dead cells. The capacity of MSCs to differentiate into specialized cell types has garnered attention for their potential therapeutic application in regenerative medicine.[170,171] Indeed, MSCs can differentiate exclusively into several mesenchymal lineages, such as chondrocytic, osteocytic and adipocytic.[172] Moreover, it has been shown that specific MSC-like cells derived from humans and mice can be stimulated to differentiate into cells of endodermal and neuroectodermal lineages, including hepatocytes, endothelial cells and neurons.[173–176]

In addition to their capacity to differentiate into specific cell types, there is substantial research interest in the immunomodulatory properties of MSCs. *In vitro* expanded MSCs have been demonstrated to regulate immune responses and alter the progression of various inflammatory diseases.[169,177,178] From a translational perspective, the capacity of MSCs to migrate preferentially to sites of inflammation or injury following intravenous infusion is particularly intriguing.[179,180]

Work by Guangwen Ren's laboratory suggests that the immunoregulatory function of MSCs is not constitutive, but instead is 'licensed' by an inflammatory microenvironment.[181,182] According to this framework, MSCs home to sites of inflammation, where proinflammatory cytokines (such as IFNγ, TNFα and IL-1β) function to induce their immunomodulatory activity.[182] The implication here is that the interaction of MSCs with the inflammatory response is malleable; higher levels of chemokines and inflammatory cytokines recruit increased numbers of MSCs, and

amplify their immunomodulatory function. Furthermore, according to this paradigm, the interaction of MSCs with immune cells may vary considerably as a disease progresses, due to the changing local cytokine milieu.

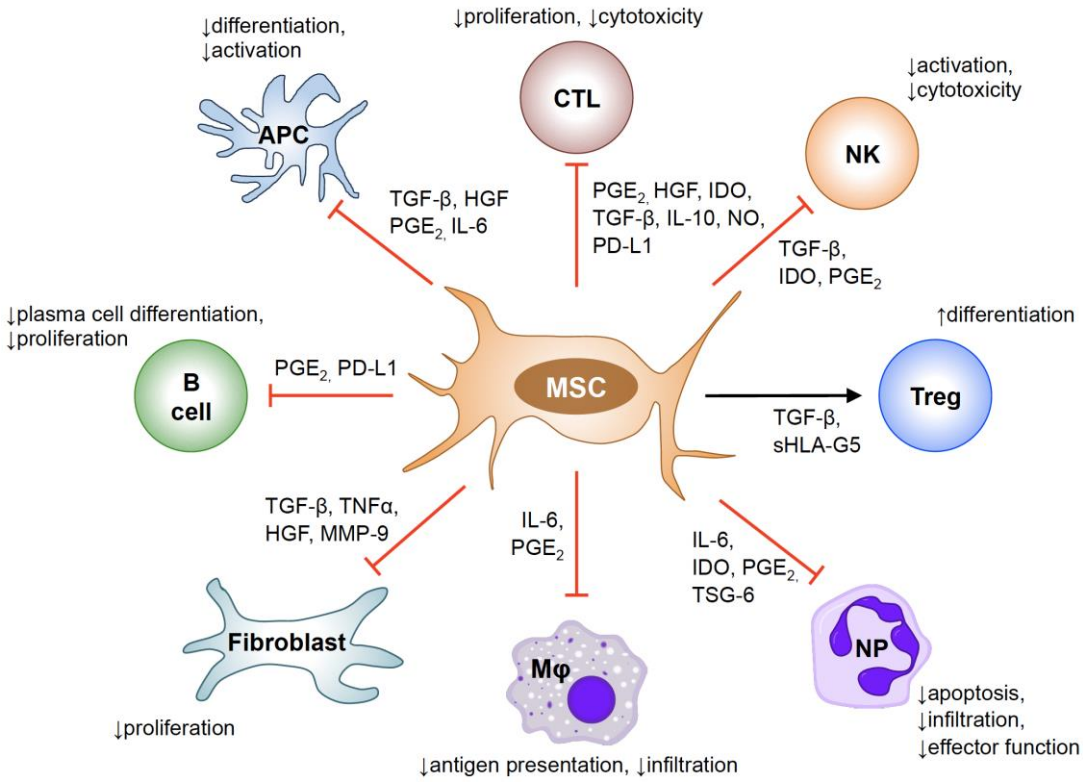

Figure 1. 7 Immunoregulatory functions of mesenchymal stem cells

MSCs modulate the immune response via both contact-dependent mechanisms and soluble factors. The proliferation and function of T cells is inhibited by MSCs. Furthermore, the activation of NK cells and APCs is limited by MSCs. The proliferation of B cells and plasma cell differentiation is suppressed by MSCs. In addition, MSCs are known to inhibit neutrophil apoptosis and limit the antigen-presenting function of macrophages. MSCs promote Treg differentiation. Diagram abbreviations: CTL, cytotoxic T cell; NK, NK cell; NP, neutrophil; Treg, regulatory T cell; Mφ, macrophage; APC, antigen-presenting cell; TSG-6, tumor necrosis factor-inducible gene 6 protein; PGE-2, prostaglandin E2; HGF, hepatocyte growth factor; TGF-β, transforming growth factor-β; IDO, indoleamine 2,3-dioxygenase; IL, interleukin; PD-L1, programmed death ligand 1; NO, nitric oxide; sHLA-G5, soluble human leukocyte antigen-G; MMP-9, matrix metallopeptidase 9; TNFα, tumor necrosis factor α. From Sahu, Foulsham et al. 2019

MSCs employ both contact-dependent mechanisms and soluble factors to regulate the immune response (Figure 1. 7). Indeed, numerous soluble factors have been demonstrated to mediate the immunomodulatory function of MSCs, including but not limited to prostaglandin E2 (PGE-2), tumor necrosis factor-inducible gene 6 protein (TSG-6), transforming growth factor β (TGF- β), indoleamine 2,3-dioxygenase (IDO) and nitric oxide (NO).[177,183–188] Cell surface molecules expressed by MSCs that have

immunoregulatory activity include several adhesion molecules (such as vascular cell adhesion molecule-1 [VCAM-1] and intercellular adhesion molecule-1 [ICAM-1]) and chemokines (such as CCR5 and CXCR3 ligands).[182,189] By recruiting immune cells, these molecules serve to co-localise MSCs with immune cells, thus creating a microenvironment with elevated levels of local immunosuppressive factors.

1.5.2. Mesenchymal stem cells in corneal transplantation

The capacity of MSCs to specifically home to the transplanted cornea has been demonstrated in experiments conducted by Omoto and colleagues.[190] In this work, the investigators generated MSCs from the bone marrow of green fluorescent protein (GFP) expressing mice, and injected these cell intravenously into allograft recipients at three hours post-transplantation. Three days following surgery, corneas and conjunctivas were harvested from grafted mice and examined using confocal microscopy. The investigators observed copious GFP^+ cells in the corneal graft, recipient bed and conjunctiva of the transplanted eye. Importantly, no GFP^+ cells were observed in the contralateral non-transplanted corneas, with only a few GFP^+ cells detected in the conjunctiva.[190] In both corneal tissue and draining lymph nodes, significantly reduced frequencies of $MHCII^+CD11c^+$ APCs in MSC-treated hosts were observed at day 14 post-transplantation by flow cytometry, compared to control hosts. Furthermore, *in vitro* experiments showed reduced maturation and function of bone marrow-derived dendritic cells when cocultured with MSCs. Analysis of the frequencies of IFNγ-secreting Th1 cells in the draining lymph nodes at day 14 post-transplantation revealed reduced Th1 frequencies in MSC-treated recipients compared to control recipients. Finally, systemic treatment with MSCs was observed to promote allograft survival compared to non-MSC-treated hosts.[190]

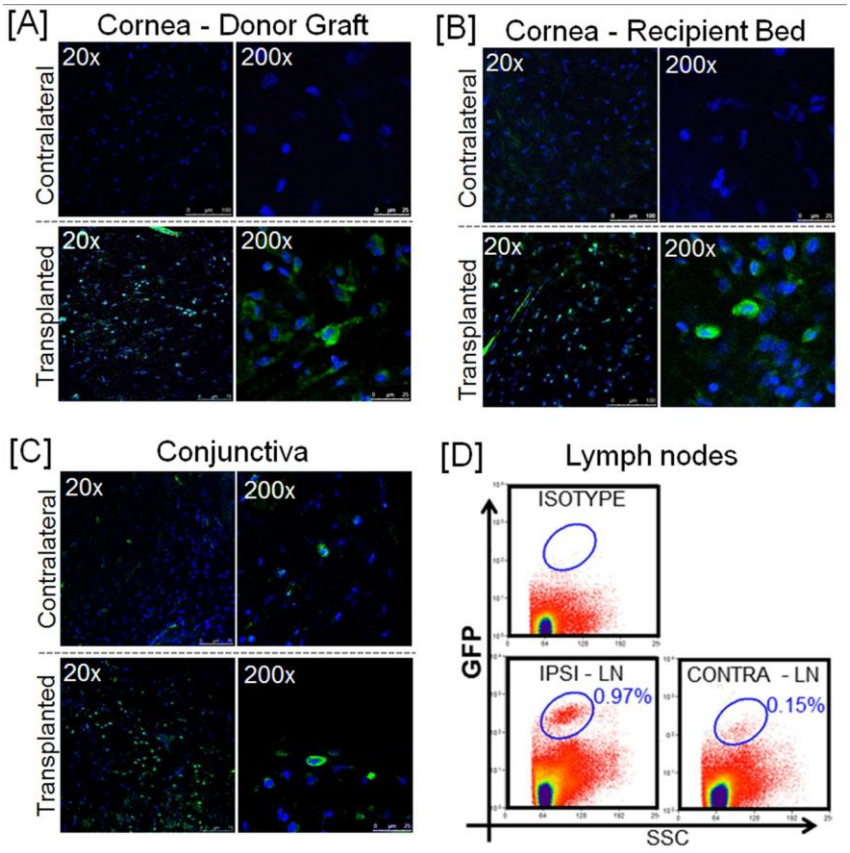

Figure 1. 8 Mesenchymal stem cells specifically home to the inflamed ocular surface and draining lymph nodes

BALB/c donor corneal buttons were grafted on C57BL/6 recipient beds. 3 hours following transplantation, 10^6 GFP$^+$ MSCs (C57BL/6) were administered via intravenous injection to the host mice. 3 days following surgery, transplanted (ipsilateral) and non-transplanted (contralateral) corneas were harvested, in addition to conjunctiva and draining lymph nodes. Harvested tissues were analyzed using confocal microscopy. Fluorescence micrographs of transplanted and contralateral **(A)** corneal grafts, **(B)** corneal recipient beds, and **(C)** conjunctiva are depicted. In panel **(D)** flow cytometric dot plots illustrate the frequencies of GFP+ MSCs in the ipsilateral (IPSI) and contralateral (CONTRA) lymph nodes of recipient mice. From Omoto et al. 2014.

The work by Omoto and colleagues[190] demonstrates that MSCs specifically home to the eye that has undergone surgical trauma, but not the contralateral (non-operated) eye. However, the investigators evaluated GFP expression at one time point only – day 3 post-transplantation. Another study by Lan and colleagues, also using GFP$^+$ MSCs, has investigated the kinetics of systemically administered MSCs using a model of corneal injury.[191] Here, the investigators demonstrate that GFP$^+$ MSCs persist in the cornea for at least 50 days following injury, with no GFP$^+$ MSCs detected in the contralateral cornea. In addition to the kinetics of exogenous GFP$^+$ MSCs, the authors observed a 2-fold increase in frequencies of circulating *endogenous* MSCs at 48 hours following injury, associated with increased levels of the stem cell chemoattractants

substance P and stromal cell-derived factor 1.[191] This work provides further evidence of MSCs homing specifically to injured corneal tissue, where they exhibit long-term survival.

In addition to the work by Omoto and colleagues, other groups have demonstrated the capacity of MSCs to promote corneal allograft survival (Table 1. 2 Models used and details of MSC administration in studies of corneal transplantation).[192–194] In a paper from Darwin Prockop's group, Oh and colleagues reported that intravenous delivery of MSCs decreased activation of antigen presenting cells in the cornea and draining lymph nodes, and resulted in prolonged allograft survival.[192] However, the investigators report that very few of the administered MSCs were observed in the cornea, with the majority of MSCs becoming trapped in the lungs, exerting their immunomodulatory activity via secretion of TSG-6. However, it is relevant to note that in Oh and colleagues' work,[192] human MSCs rather than MSCs expanded from murine bone marrow were administered. Thus, this apparent inconsistency between reports may be related to potential issues of engraftment and integration when cells are administered across the xeno-species barrier, with human MSCs being administered in a mouse model of corneal transplantation.[171]

Author	Model	Tissue source of MSCs	Number of MSCs/animal and route of administration
Omoto et al., 2014[190]	Orthotopic allogeneic corneal transplantation (mouse): 2mm diameter corneal cup from donor (C57BL/6) grafted onto 1.5mm diameter prepared host bed (BALB/c) using 8 interrupted nylon 11-0 sutures	Murine bone marrow (BALB/c)	10^6 cells in 100 µL 0.9% NaCl via IV injection
Oh et al., 2012[192]	Orthotopic allogeneic corneal transplantation (mouse): 2mm diameter corneal cup from donor (C57BL/6) grafted onto 1.5mm diameter prepared host bed (BALB/c using 8 interrupted nylon 11-0 sutures	Human MSCs (from Center for the Preparation and Distribution of Adult Stem Cells)	10^6 cells in 100 µL HBSS via IV injection
Jia et al., 2012[193]	Orthotopic allogeneic corneal transplantation (rat): 3.5mm diameter corneal cup from donor (Wistar) grafted onto a 3mm diameter prepared host bed (Lewis) using 8 interrupted nylon 10-0 sutures	Rat bone marrow (Wistar)	$5x10^6$ cells in 1ml PBS via IV injection
Treacy et al., 2014[194]	Orthotopic allogeneic corneal transplantation (rat): 3mm diameter corneal cup from donor (Dark Agouti) grafted onto a 2.5mm diameter prepared host bed (Lewis) using 8-10 interrupted nylon 10-0 sutures	Rat bone marrow (Lewis, Dark Agouti or Wistar rats)	10^6 cells in 1ml PBS via IV injection

Table 1. 2 Models used and details of MSC administration in studies of corneal transplantation
From Sahu, Foulsham et al. 2019.

Jia and colleagues employed a rat model of corneal transplantation to investigate how systemic delivery of MSCs modulates the alloimmune response.[193] The investigators demonstrate that, in addition to limiting the generation of graft-attacking Th1 cells, administration of MSCs also significantly increased frequencies of $CD4^+CD25^+Foxp3^+$ Tregs relative to vehicle-treated hosts.[193] Indeed, Treg expansion following MSC administration in a rat model of corneal allotransplantation has also been reported by Treacy and colleagues.[194] The study by Treacy and colleagues evaluated the immunomodulatory effects of administering MSCs from 3 different sources in a fully allogeneic model of corneal transplantation (Dark Agouti donors and Lewis recipients). Thus, MSCs sourced from Lewis rats were syngeneic, MSCs sourced from Dark Agouti were allogeneic, and MSCs sourced from Wistar Further rats were third party.[194] Interestingly, the investigators demonstrated that corneal transplant survival

was prolonged when either allogeneic or third party MSCs were administered, but all grafts were rejected when syngeneic MSCs were injected. This compares with a rejection rate of 80% in untreated recipients. In addition to increased frequencies of splenic Tregs in the allogeneic MSC-treated and third party MSC-treated groups, the authors also report decreased corneal infiltration of natural killer T cells in these groups, providing evidence of both local and systemic immunomodulation.[194]

1.5.3. Hepatocyte growth factor

Hepatocyte growth factor (HGF) is a pleiotropic cytokine that acts via the receptor *c-met* to promote the proliferation and morphogenesis of epithelia.[195,196] Over recent years, studies utilizing tissue-specific knockout mice have demonstrated that the HGF/*c-met* signalling pathway is also critically involved in protecting against fibrosis and regulating both acute and chronic inflammation.[195,197] Indeed, HGF has been shown to modulate immune responses in a variety of models of autoimmunity including inflammatory bowel disease, collagen-induced arthritis and experimental autoimmune encephalomyelitis.[195]

Previous work has suggested that the anti-fibrotic and anti-inflammatory function of MSCs is, in part, mediated by production of HGF.[198–201] Using a model of mechanical corneal injury in which MSCs were administered intravenously at 1 hour post-injury, Mittal and colleagues have reported that MSC-derived HGF functions to restore corneal transparency following injury.[199] The investigators observed that an inflammatory milieu drives MSCs to secrete increased levels of HGF, and furthermore that the capacity of MSCs to restore corneal transparency is contingent on their expression of HGF. The investigators also evaluated the therapeutic efficacy of topical HGF alone (independent of MSC administration) following injury, and found that topical HGF was sufficient to restore corneal transparency.[199] In addition to decreased opacity scores in the HGF-treated group, immunohistochemistry analyses of corneal cross-

sections immunostained for the fibrosis marker α-smooth muscle actin (αSMA) revealed that treatment with HGF abrogated the increased αSMA expression observed following injury. Finally, harvested corneas analysed for their mRNA expression of IL-1β and TNF-α revealed substantially reduced expression in HGF-treated corneas relative to albumin-treated controls.[199]

1.6. Dry eye disease

1.6.1. Dry eye disease epidemiology

Dry eye disease (DED; also known as keratoconjunctivitis sicca) is defined as 'a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play aetiological roles'.[202] Symptoms that are commonly described by patients with DED include dryness, foreign body sensation, itching, irritation and light sensitivity.

DED is an extremely common chronic condition that markedly reduces patient quality of life.[203–205] Indeed, a recent epidemiological study based on data from a large, all-age United States health care system (approximately ten million patients) estimates a DED prevalence of 5.3% in the general population.[206] Age is a recognized risk factor for DED, with the prevalence increasing from 0.2% for ages 2-17 years, to 11.7% for those older than 50 years.[206] Estimates of DED prevalence for the population aged greater than 65 range from 12.9 to 14.6%.[206–208] Thus, as populations in developed countries grow older relative to historical norms, the burden of DED-associated morbidity is anticipated to increase.

Female sex is also a risk factor for DED, with an overall prevalence of 7.8% among females compared to 3.0% among males.[206] Other risk factors for DED include being of Asian race, Meibomian gland dysfunction, Sjogren syndrome, contact lens use, hematopoietic stem cell transplantation, refractive surgery, visual display use, environmental exposures (e.g. low humidity environments) and vitamin deficiency.[209] Although the pathogenesis of DED is not fully understood, there is strong evidence implicating inflammation as a core driver of disease. Indeed, the general thesis of the

Dry Eye Workshop II is that desiccating stress initiates DED, which is perpetuated by a vicious circle of ocular surface inflammation.[210]

1.6.2. Dry eye disease pathogenesis

In DED, ocular surface hyperosmolarity induces a proinflammatory environment at the ocular surface, and this milieu triggers the activation of antigen presenting cells (APCs) (Figure 1. 9). APCs egress from the cornea and are trafficked along afferent lymphatics to the draining lymphoid tissue.[50,211,212] In the lymph nodes, APCs induce effector helper T cell differentiation from naïve T cells, which subsequently migrate via efferent blood vessels to the ocular surface, where they release proinflammatory factors.[50,211,212] Accordingly, DED immunopathogenesis can be understood as a vicious circle, in which a proinflammatory microenvironment promotes the infiltration of pathogenic immune cells, which in turn damage the ocular surface and recruit additional inflammatory cells.

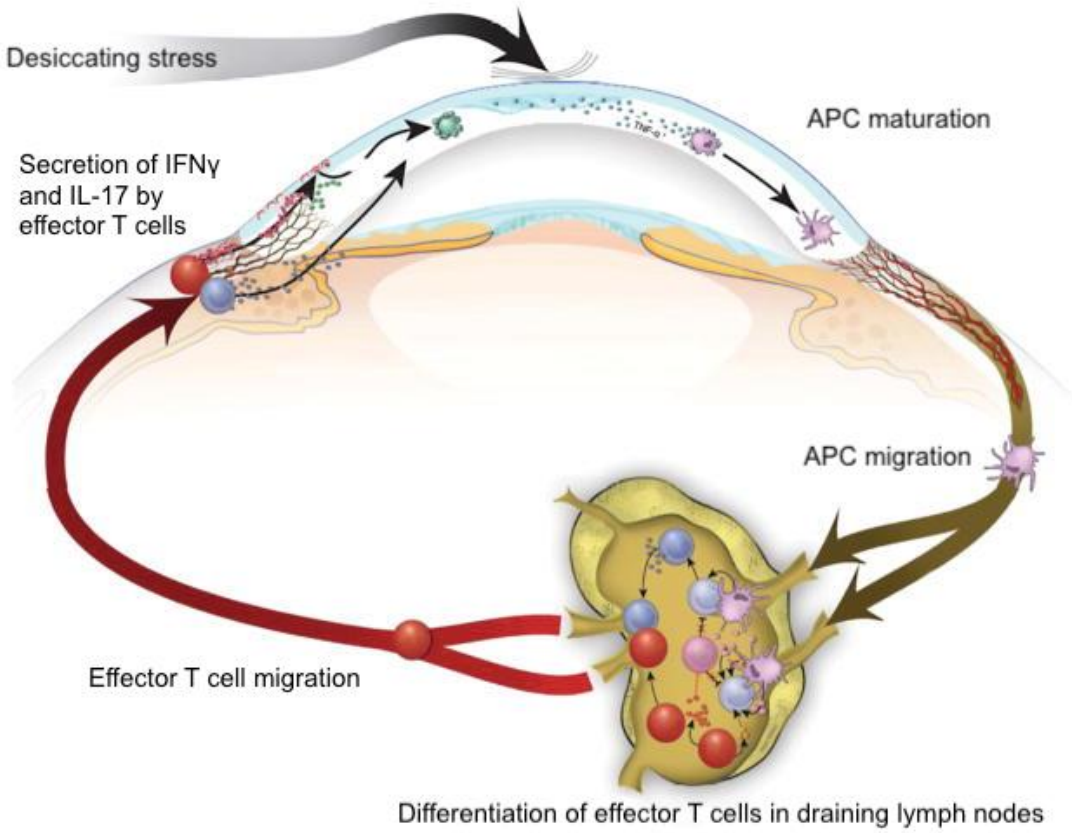

Figure 1. 9 Dry eye disease immunopathogenesis

Tear hyperosmolarity is induced by desiccating stress, which results in the activation of mitogen-activated protein kinase signaling pathways, thereby promoting the production of inflammatory cytokines such as IL-1β, TNF-α and IL-6. This pro-inflammatory environment drives the maturation of antigen-presenting cells (APCs), which subsequently are trafficked via afferent lymphatic vessels to draining lymphoid tissue, where they promote the differentiation of interferon-γ-secreting T helper 1 (Th1) and interleukin-17-secreting T helper 17 (Th17) cells from naïve T cells. Following this, Th1 and Th17 cells migrate via efferent blood vessels to the ocular surface. Regulatory T cells antagonize the differentiation of Th1 and Th17 cells in the lymphoid tissue. On reaching the ocular surface, effector T cells promote the production of proinflammatory cytokines, matrix metalloproteinases, chemokines, cell adhesion molecules, and prolymphangiogenic factors that damage the corneal epithelium and promote the infiltration of additional immune cells. Adapted from Stevenson et al. 2013

1.6.2.1. Inflammatory signals

Desiccating stress leads to a hyperosmolar environment at the ocular surface, which in turn activates mitogen-activated protein kinases (MAPK), including c-Jun N-terminal kinase and extracellular signal-related kinase.[213–215] MAPK signalling pathways activate the master regulator nuclear factor κ B (NFκB), which triggers a cascade of downstream mediators and cellular signals including proinflammatory cytokines, chemokines, and matrix metalloproteinases (MMPs).[210,211] Indeed, the expression of

MMP-1, MMP-3, MMP-9 and MMP-13 by corneal epithelial cells has been shown to be increased following exposure to hyperosmolar stress.[216] Upregulated MMP-9 production by corneal epithelial cells has been shown to be associated with disruption of the corneal epithelium.[217] Moreover, interrupting MMP-9 signalling in an experimental model of DED with MMP-9 knockout mice has been shown to ameliorate disease severity, implicating MMP-9 in the pathogenesis of desiccation-induced corneal epitheliopathy.[218] MMP-9 is also known to be involved in the activation of IL-1β into its mature, biologically active form.[219] MMPs are understood to increase DED severity by promoting the degradation of corneal extracellular matrix and causing epithelial cell loss.[211,220,221] The rationale for treating DED with doxycycline, a tetracycline antibiotic, is that doxycycline inhibits MMP activity.[221,222]

1.6.2.2. Recruitment signals

The ensuing pro-inflammatory milieu at the ocular surface (including heightened expression of the cytokines IL-1β, TNF-α and IL-6) amplifies itself and promotes the activation of immature, resident APCs, which subsequently egress from the cornea and migrate to the draining lymphoid tissues.[210,211] Indeed, in an experimental model of DED, blockade of signalling via IL-1 receptor (IL-1R) using IL-1R knockout mice has been shown to ameliorate disease severity and attenuate production of inflammatory cytokines.[223]

Expression of chemokines and endothelial adhesion molecules facilitate the homing of immune cells. Chemokines that are upregulated during ocular surface inflammation include CCL3, CCL4, CCL5, CXCL8, CXCL9, CXCL10, CXCL11 and CX3CL1.[210,224–227] CCL3 and CCL4 are expressed by macrophages and serve to recruit inflammatory cells (including neutrophils) and promote the production of inflammatory cytokines.[211] CCL5 (RANTES) is chemotactic for various leukocytes, and contributes toward NK cell activation.[228] CXCL8 is a neutrophil chemoattractant, and is produced by cells with toll-

like receptors including epithelial cells and macrophages. CXCL9, CXCL10 and CXCL11 bind the chemokine receptor CXCR3 and function as T cell chemoattractants.[211]

Expressed at the cell surface, cell adhesion molecules promote cell migration by interacting with components of other cells and the extracellular matrix. Intracellular adhesion molecule-1 (ICAM-1) is expressed by corneal and conjunctival epithelium in DED, as well as by vascular endothelium.[229] Inflammatory cells that express the ligand for ICAM-1, integrin leukocyte functional antigen 1 (LFA-1), undergo rolling, transmigration and activation following interaction of ICAM-1 and LFA-1.[230] Experiments in which ICAM-1 was targeted with monoclonal antibodies have established that ICAM-1 expression promotes inflammation in DED.[231] Vascular cell adhesion molecule is expressed by vascular endothelial cells, and binds to very late antigen 4. Topical blockade of very late antigen 4 has been shown to decrease DED severity in an animal model, and has also been demonstrated to suppress the molecular mediators of DED.[232]

1.6.2.3. Antigen-presenting cells in dry eye disease

The contribution of adaptive immunity to the sustained ocular surface inflammation observed in DED has been demonstrated in multiple studies.[233–236] Further evidence is derived from increased frequencies of $CD4^+$ T cells at the ocular surface in patients with DED, as well as the fact that topical cyclosporine ameliorates clinical disease.[237,238] As discussed in Chapter 1, the generation of an adaptive immune response depends on the processing of antigens by APCs, that are then presented to naïve T cells in the regional draining lymphoid tissue to trigger the differentiation and clonal expansion of antigen-specific effector T cells. Yet unlike corneal transplantation, where the antigens are known,[79] it is not clear which antigens initiate the adaptive immune response in DED. Nevertheless, studies employing mouse models of DED

have demonstrated increased corneal infiltration of $CD11b^+$ cells following exposure to desiccating stress, with increased expression of MHCII.[239–241] Notably, mice that are depleted of ocular surface APCs and macrophages that are exposed to desiccating stress exhibit reduced $CD4^+$ T cell infiltration relative to wild-type control animals.[242] These observations are consistent with *in vivo* confocal microscopy studies that demonstrate increased frequencies of dendritic-like cells in the corneas of patients with DED.[243,244]

1.6.2.4. Effector T cells in dry eye disease

T helper 17 (Th17) cells have emerged as the principal effector immune cells in DED.[234,235,245] This is particularly notable given the key immunopathogenic role of these cells in autoimmune conditions such as uveitis/scleritis,[246] inflammatory bowel disease,[247] rheumatoid arthritis[248] and multiple sclerosis.[249] Using an experimental model of DED, Chauhan and colleagues have demonstrated that Th17 cells are relatively resistant to suppression by Tregs in DED.[245] The investigators show that *in vivo* blockade of IL-17 reduces disease severity, limits the expansion of Th17 cells and restores Treg function.[245] The importance of Th17 cells in driving DED pathogenesis is corroborated by work by De Paiva and colleagues, who also show that antibody neutralization of IL-17 ameliorates DED.[235] In this work, the investigators demonstrate that desiccating stress increased expression of Th17-associated genes (including IL-6, IL-23, TGF-β1, TGF-β2, IL-23R, IL-17A) by ocular surface tissues using an experimental mouse model. Furthermore, in conjunctival samples obtained from human subjects with DED, increased gene expression of the Th-17 inducers IL-23 and IL-17A was observed.[235] Of note, IL-17 promotes corneal lymphangiogenesis via VEGFD/C-VEGFR3 signaling, thus upregulating the inflammatory response at the ocular surface by facilitating the migration of immune cells.[143]

Work by Chen and colleagues has demonstrated an important role for IFNγ in DED pathogenesis.[250] Specifically, the authors showed that there is a significant population of $CD4^{+}IL\text{-}17A^{+}IFN\gamma^{+}$ ('double-positive' Th17/1) cells, that are derived from Th17 precursors. In contrast to adoptive transfer of Th1 cells, adoptive transfer of double-positive Th17/1 cells to naïve mice induced similar corneal epitheliopathy as transfer of Th17 cells alone. The investigators identified that IL-12 and IL-23 are necessary for the *in vivo* transition of Th17 cells into double-positive Th17/1 cells.[250] Furthermore, the importance of IFNγ expression by Th17/1 cells was demonstrated in experiments using IFNγ-deficient Th17 cells, the adoptive transfer of which resulted in less severe disease as compared to double-positive Th17/1 cells.[250]

Much of the experimental data on DED pathogenesis has been derived from models of acute DED, in which mice are exposed to desiccating stress and/or treated with antimuscarinic agents for a finite period (typically 2 weeks).[233,234,251,252] Yet this contrasts with clinical DED, which usually is encountered as a chronic condition. Interestingly, when mice are exposed to desiccating stress for a period of 2 weeks and then housed in a standard vivarium, a low-grade corneal epitheliopathy is evident by corneal fluorescein staining for several months.[253] This chronic phase of the disease was observed to be mediated primarily by Th17 immunity. Notably, a significant population of memory Th17 cells ($CD4^{+}CD62L^{-}CD44^{hi}$) was recovered from mice during the chronic phase (Figure 1. 10), and these memory Th17 cells were shown in adoptive transfer experiments to be more pathogenic (i.e. greater disease severity and rate of disease progression) relative to effector Th17 cells.[253]

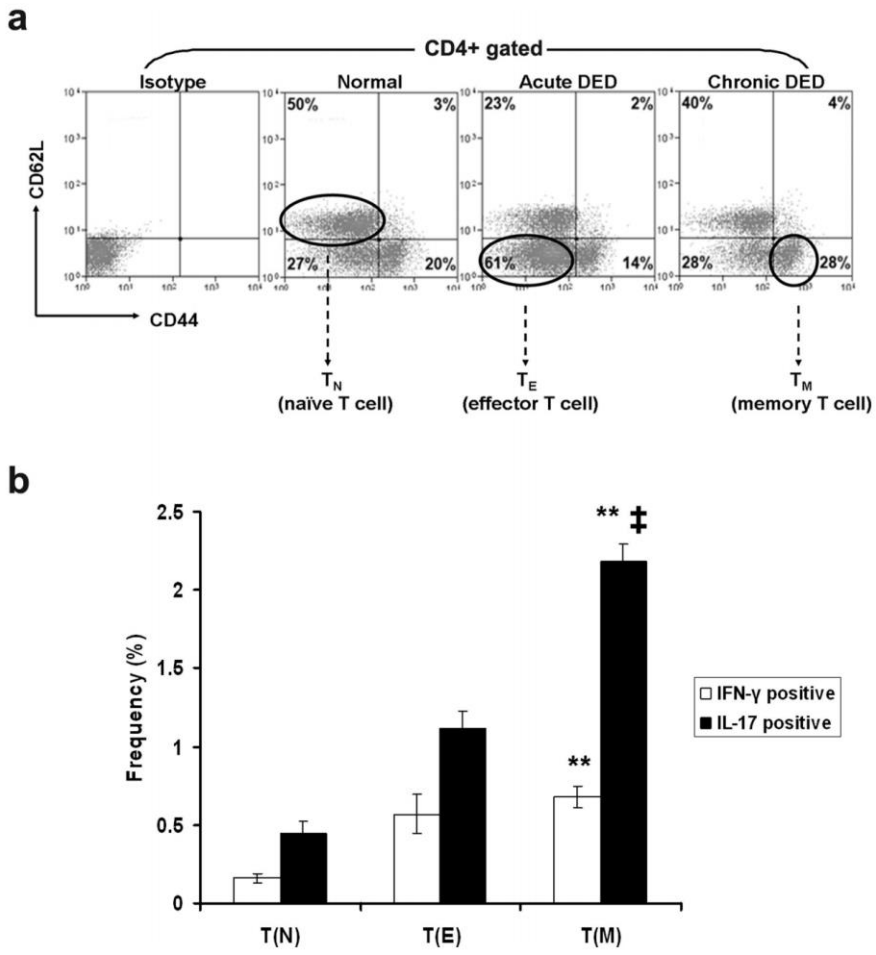

Figure 1. 10 Memory Th17 population in chronic dry eye disease

T cells derived from chronic DED mice demonstrate an amplified memory Th17 population. Draining lymph nodes and spleens were harvested from naïve, acute DED (14 days desiccating stress) and chronic DED (14 days desiccating stress, followed by 112 days room air). Data shown are derived from spleen, with similar results for lymph nodes (not shown). **(a)** Expression of CD62L and CD44, gated on $CD4^+$ cells, is presented. Four populations of T cells are apparent: $CD62L^+CD44^{lo}$ naïve T cells (T_N), $CD62L^-CD44^{lo}$ effector T cells (T_E), $CD62L^+CD44^{hi}$ central memory T cells, and $CD62L^-CD44^{hi}$ effector memory T cells (T_M). The frequencies of each cell population are shown. **(b)** IFNγ and IL-17 expression on T_N sorted from normal, T_E sorted from acute, and T_M sorted from chronic DED was evaluated. Frequencies of $IFN\gamma^+$ or $IL\text{-}17^+$ cells in each of these populations are shown. Data depicted are mean +/- SEM. ** $p < 0.01$. From Chen et al. 2014

Memory Th17 cells have also been demonstrated to predispose toward severe DED exacerbations.[254] Upon re-exposure to desiccating stress, mice that have previously been challenged in the same environment exhibit an increase in corneal fluorescein staining scores at least twice that of mice undergoing primary challenge.[254] Interestingly, similar findings have been observed in experimental autoimmune encephalitis, with memory Th17 cells implicated in promoting earlier onset and increased severity of clinical disease after rechallenge.[255]

1.6.2.5. Regulatory T cells in dry eye disease

As discussed in Chapter 1, Foxp3$^+$ Tregs are critical modulators of immune homeostasis. The importance of Tregs in suppressing autoimmunity is evidenced both by the fatal human disorder of immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome,[40] as well as by murine models,[256] showing the effects of FOXP3 mutations. Experimental studies employing desiccating stress have elucidated the importance of Tregs in curbing the inflammatory response in DED.

Depletion of CD4$^+$CD25$^+$Foxp3$^+$ Tregs has been demonstrated to exacerbate corneal epitheliopathy in mice subsequently exposed to a dry environment.[257] The tear concentration of the proinflammatory cytokines IFNγ, TNFα and IL-12 has been shown to be increased in recipient mice following adoptive transfer of CD4$^+$ T effector cells derived from mice exposed to desiccating stress.[258] However, this phenomenon was abrogated when Tregs were co-transferred with CD4$^+$ effector T cells at a ratio of 1:1.[258] Interestingly, it has been shown that Tregs isolated from DED mice have impaired suppressive function.[245] Indeed, although the frequencies of Tregs in naïve and DED mice are comparable, Tregs sourced from DED mice have been shown to be less effective at suppressing T cell proliferation.[245] Notably, Tregs isolated from DED mice are particularly inept at suppressing the proliferation of Th17 cells. Indeed, the primary proliferating subset of primed T cells co-cultured with DED Tregs has been shown to be Th17 cells.[245]

As described earlier in this chapter, ageing is a major risk factor for DED.[206] It has been suggested that Tregs may develop function-specific impairments with increased age.[259] For example, aged Tregs may exhibit reduced restraint of IL-17 immunity, yet still be competent in modulating other inflammatory responses (e.g. against neoplastic or infectious diseases).[259] In a study employing NOD.B10.H2^b mice (which

spontaneously develop a dry eye phenotype), Coursey and colleagues demonstrate that Treg frequencies increase in ageing despite increased severity of lacrimal gland pathology.[260] However, the authors show that aged Tregs lose suppressive function despite maintaining Foxp3 expression. Furthermore, a portion of $Foxp3^+$ Tregs were observed to express IFNγ and IL17, and these cells were shown to be highly pathogenic in adoptive transfer experiments.[260] These results suggest that, beyond losing their functional competence, $Foxp3^+$ Tregs may in fact produce inflammatory cytokines and contribute to the pathogenesis of age-related DED.

1.7. Aims

Excessive inflammation of the cornea impairs visual function and is potentially blinding. Much progress has been made in identifying features at the ocular surface that foster its particularly tolerant immune microenvironment, yet there are gaps in our current knowledge.[50] The aims of this thesis are concerned with investigating immunoregulation at two specific phases of the adaptive immune response – antigen recognition and generation of memory (as highlighted in Figure 1. 11).

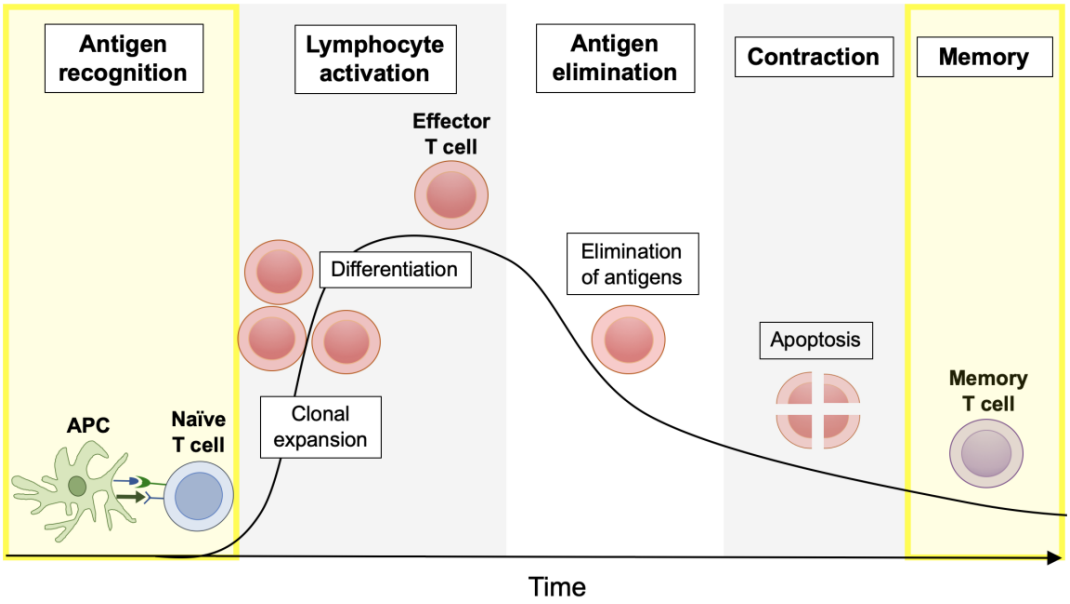

Figure 1. 11 Phases of the adaptive immune response

The adaptive immune response consists of distinct phases. The adaptive immune response is initiated when antigens are ingested by immature antigen-presenting cells, which subsequently undergo maturation. The mature antigen-presenting cells migrate to a regional lymph node, where they activate lymphocytes. Effector lymphocytes eliminate antigen. During contraction, antigen-stimulated lymphocytes undergo apoptosis, with the exception of subset of antigen-specific cells that form the memory pool. The phases of the immune response that this thesis will focus on (antigen recognition and memory) are highlighted.

In order to investigate immunoregulation at the antigen recognition phase of the adaptive immune response, a well-established murine model of corneal transplantation was used.[164,165,168,261] Unique among models of transplantation, this model allows the continuous assessment of the alloimmune response (by evaluating graft opacity) without requiring sacrifice of the host. In order to investigate the generation of the memory pool, a well-established murine model of dry eye disease was

employed.[250,262–264] Previous work has demonstrated that a sizeable pool of memory T cells is formed following primary challenge using this model, and that this memory pool predisposes to severe and accelerated disease upon subsequent rechallenge.[253,254]

My aims were to utilize these two models to further characterize specific features of the ocular inflammatory response during the antigen recognition and memory generation phases of the adaptive immune response. Furthermore, I sought to evaluate potential therapeutic strategies to decrease detrimental corneal inflammation in these settings. Specifically:

- To characterize the effect of the purinergic receptor antagonist oxidized adenosine triphosphate on corneal allograft survival
- To determine the role of MSC-derived hepatocyte growth factor in the promotion of corneal allograft survival
- To determine the contribution of memory Th17 cells to the severe dry eye disease observed in aged mice

CHAPTER 2. Materials and methods

2.1. Reagents

Laboratory supplies and powdered reagents were sourced from established suppliers.

2.2. General *in vivo* methods

2.2.1 Animals

Animals were housed in a secure, pathogen-free vivarium at the Schepens Eye Research Institute Animal Facility, Massachusetts Eye & Ear, USA. Animals were treated according to the guidelines detailed by the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. All animal procedures were reviewed and approved by the Schepens Eye Research Institute Animal Care and Use Committee.

For corneal transplantation studies, six- to eight-week-old male C57BL/6 mice were used as donors and similarly aged male BALB/c mice were used as hosts. For dry eye disease studies, six- to eight-week-old (young) and twelve- to fourteen-month-old (aged) female C57BL/6 mice were used. For corneal injury studies, six- to-eight-week-old male C57BL/6 mice were used. All mice were purchased from Charles River Laboratories (Wilmington, MA, USA).

2.2.2 Anaesthesia and analgesia

For *in vivo* procedures including corneal transplantation, corneal suture removal and corneal injury, mice were anaesthetized by a single intraperitoneal injection of ketamine hydrochloride (100mg/kg) combined with xylazine hydrochloride (20mg/kg). In addition, topical proparacaine 0.5% ophthalmic solution was administered to the ocular surface at 15-minute intervals until the end of the procedure. For analgesia, buprenorphine hydrochloride (0.1mg/kg) was administered via subcutaneous injection

immediately following the procedure and was re-administered every 8-12 hours for 48 hours post-operatively.

2.3. Corneal transplantation

The mouse model of corneal transplantation employed in these studies is well-established, and has been used by multiple groups for a number of decades.[128,157,265,266] In this model of ocular alloimmunity, approximately half of the grafts undergo rejection in the first 4 weeks post-operatively, with the remaining half surviving indefinitely. One major advantage of this model is that it permits continuous assessment of the alloimmune response (by evaluating graft opacity) without requiring host sacrifice. Other advantages include the relative lack of expense relative to larger animals, the availability of a wide range of reagents and the availability of mice deficient in specific genes. Disadvantages of the model include the fact that the diagnosis of rejection is based on loss of graft clarity and is inherently subjective. The surgical technique employed by our laboratory has been described previously.[267]

2.3.1 Surgical technique: donor preparation

The donor mouse was euthanized using carbon dioxide. A 2 mm diameter trephine (HealthLink, Jacksonville, FL, USA) was used to mark the central cornea (Figure 2. 1). A 30G needle was used to tunnel into the anterior chamber through part of the 2mm diameter corneal marking. Through the tunnel, ocular viscoelastic (Bausch & Lomb, Tampa, FL, USA) was injected into the anterior chamber. Vannas scissors were used to cut along the 2mm diameter corneal marking, resulting in the excision of a 2mm diameter donor corneal button. The corneal button was transferred into sterile PBS in a culture dish.

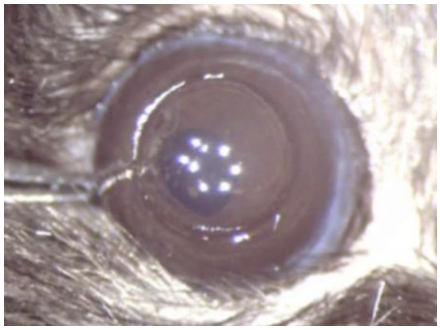

Figure 2. 1 Donor corneal button
From Inomata et al. 2019

2.3.2 Recipient preparation

Recipient mouse was anaesthetized with ketamine (120 mg/kg) and xylazine (20 mg/kg) injected via intraperitoneal injection using a 25G needle. The anaesthetized animal was laid in a lateral recumbent position, and whiskers and eye lashes were cut. The ocular surface was washed with sterile PBS and cleaned using eye spears (Beaver Visitec International, Waltham, MA, USA). Proparacaine eye drops (0.5%; Bausch & Lomb, Tampa, FL, USA) were applied to the corneal surface. After 2 minutes, tropicamide eye drops (Bausch & Lomb, Tampa, FL, USA) were applied to the corneal surface. When adequate pupillary dilation was achieved (approximately 2 minutes), excess eye drops were dried from the ocular surface using eye spears.

A 1.5 mm trephine (HealthLink, Jacksonville, FL, USA) was used to mark the central cornea. Similar to the technique employed in harvesting the donor cornea button, a 30G needle was used to tunnel through the corneal marking into the anterior chamber, the depth of which was maintained by the subsequent injection of ocular viscoelastic. The 1.5 mm central disk of the host cornea was excised using Vannas scissors.

The 2 mm donor button is secured to the 1.5 mm host bed using 8 interrupted 11-0 nylon sutures (MANI, Tochigi, Japan). After placing the first 4 sutures at 12, 3, 6 and 9 o'clock, ocular viscoelastic was injected into the anterior chamber to maintain its structure. Once the donor cornea is secured by 8 sutures (Figure 2. 2), the anterior chamber was washed with PBS to remove residual ocular viscoelastic. Triple antibiotic eye ointment (bacitracin zinc, neomycin, polymyxin B sulfate; Bausch & Lomb, Tampa, FL, USA) was applied to the operated eye. Importantly, triple antibiotic eye ointment was also applied to the contralateral eye

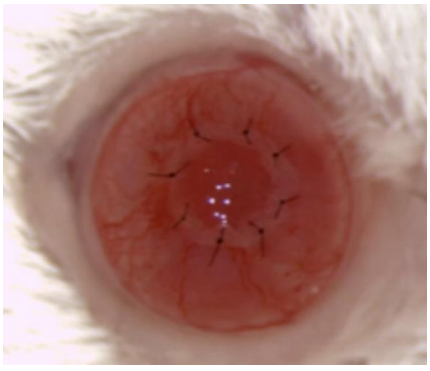

Figure 2. 2 Recipient bed and graft
From Inomata et al. 2019

during the procedure to maintain a moist ocular surface. 8-0 nylon sutures (MANI, Tochigi, Japan) were used to perform a tarsorrhaphy over the grafted eye, which was removed at 24 hours post-operatively. Buprenorphine hydrochloride (0.1mg/kg) was administered via subcutaneous injection immediately following the procedure and was re-administered every 8-12 hours for 48 hours post-operatively. The 8 interrupted 11-0 nylon sutures were removed at day 7 post-transplantation.

2.3.3 Clinical assessment

Graft survival was assessed for 8 weeks following transplantation. Graft opacity was determined on a weekly basis using a slit-lamp biomicroscope according to an established scoring system (range 0-5+; [Table 2. 1]).[128,163,164,268,269] Immune rejection was defined as a score of more than 2 at two consecutive weekly examinations (i.e. a degree of opacity that obscures iris details). Grafts with evidence of technical difficulties (including infection, loss of the anterior chamber, infection) were excluded from further analysis. In addition, to exclude grafts failing for reasons other than immune rejection, grafts with scores of more than 2 at post-operative day 14 were excluded from analysis.

Score	Clinical observation
0	Clear
1	Minimal superficial opacity, iris vessels visible
2	Minimal stromal opacity, iris vessels visible
3	Moderate stromal opacity, total pupil margin visible
4	Intense stromal opacity, portion of pupil margin visible
5	Maximal stromal opacity, anterior chamber not visible

Table 2. 1 Standardized scoring system for corneal graft opacification
From Sonoda and Streilein 1992.

2.3.4 Anticipated results

BALB/c mice transplanted orthotopically with fully allogeneic C57BL/6 corneas, as described above, results in approximately 50% graft rejection, while the remainder of grafts enjoy indefinite survival.[152]

2.3.5 Surgical success rate

Before beginning any *in vivo* experiments employing allogeneic corneal transplantation, I practiced my surgical technique with syngeneic transplantation (i.e. BALB/c donors with BALB/c hosts). Since 100% grafts are expected to survive under these conditions, I was able to practice until I had eliminated other causes of graft failure. I reached 100% survival after 40 syngeneic grafts. Issues that I overcame during these practice transplantations included:

- Instability of 11-0 needle in needle holder. I tried a number of different needle holders and forceps before identifying a pair that provided adequate stability.
- Minimizing trauma to endothelium during transplantation. I tried both jewellers' forceps and toothed microforceps to hold the donor button when passing the needle through the tissue. I recognized that if the donor button was not securely held on the first attempt, repositioning the forceps was causing unnecessary trauma to the donor endothelium. Eventually I settled on jewellers' forceps, positioning the forceps parallel rather than perpendicular to the proximal border of the donor button.

2.4. Corneal injury

In my corneal injury experiments, a handheld motor brush (AlgerBrush II, Alger Company, Lao Vista, TX, USA) was used to mechanically remove the corneal epithelium and anterior stroma (Figure 2. 3). It is important to note that this differs from epithelial debridement models, which use a similar mechanical burr to remove the epithelium but leave the basement membrane intact.[199,200,270,271]

Mice were anaesthetized with ketamine (120 mg/kg) and xylazine (20 mg/kg) as described in Section 2.3. The ocular surface was washed with sterile PBS and cleaned using eye spears (Beaver Visitec International, Waltham, MA, USA). Proparacaine eye drops (0.5%; Bausch & Lomb, Tampa, FL, USA) were applied to the corneal surface. The central cornea was marked using a 2 mm trephine (HealthLink, Jacksonville, FL, USA). Using the tip of a handheld motor brush (AlgerBrush) the corneal epithelium and the anterior third of the corneal stroma were removed. Upon completion of the procedure, triple antibiotic eye ointment (bacitracin zinc, neomycin, polymyxin B sulfate; Bausch & Lomb, Tampa, FL, USA) was applied to the operated eye. Buprenorphine hydrochloride (0.1mg/kg) was administered via subcutaneous injection immediately following the procedure and was re-administered every 8-12 hours for 48 hours post-operatively.

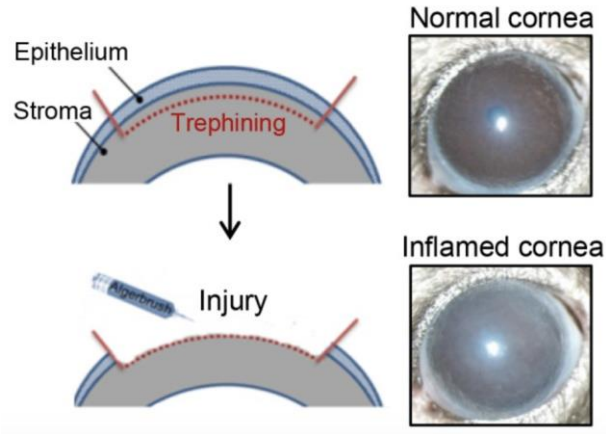

Figure 2. 3 Diagram showing mouse model of corneal injury
From Mittal et al. 2018.

2.5. Dry eye disease

The mouse model of dry eye disease (DED) employed in this work is well-established, with variations of the model being used by multiple groups around the world.[257,272–274] Either a controlled-environment chamber (which permits the continuous regulation of temperature, humidity and airflow) or an air blower are used to produce desiccating environmental conditions. The exposure to dry conditions results in increased tear evaporation and triggers an innate immune response, resulting in the activation of NF-κB and MAPK signalling pathways in ocular surface immune and epithelial cells, resulting in the secretion of pro-inflammatory cytokines, matrix metalloproteinases

(MMPs) and chemokines.[66,214,220,252,272,275] This pro-inflammatory milieu drives the maturation of antigen-presenting cells, which migrate via afferent lymphatics to the draining lymph nodes, where they induce the differentiation of naïve Th0 cells into effector Th17 and Th1 cells.[211] Subsequently, effector Th17 and Th1 cells home to the ocular surface via efferent blood vessels across a chemokine gradient.[211] At the ocular surface, these cells produce proinflammatory cytokines, MMPs and cytokines that amplify the infiltration of pathogenic immune cells.[211]

It is important to emphasize that the infiltrating T cells observed in this model are *disease specific.* Experiments have demonstrated that DED can be induced in T-cell-deficient mice by the adoptive transfer of $CD4^+$ T cells isolated from the draining lymph nodes of mice previously exposed to desiccating stress.[253,257] It is also important to emphasize that this model represents *antigen-driven disease.* Depletion of ocular surface APCs using the compound clodronate has been shown to protect mice against ocular inflammation despite exposure to desiccation, and $CD4^+$ T cells derived from clodronate-treated mice fail to induce inflammation in recipient T-cell-deficient mice.[242]

The desiccating stress murine model has been shown to recapitulate key features of human DED,[276] which are summarized below (

Table 2. 2).

Disease marker/parameter	Mouse model	Human DED
Increased innate inflammatory mediators (cytokines, MMPs, chemokines)	66,214,220,252,272,275	217,277,278
Increased ICAM1 expression on lacrimal gland and conjunctiva	231,277	279
Increased epithelial apoptosis	280,281	282
Conjunctival goblet cell loss	283,284	285,286
Dendritic cell maturation	242,287	282,288
$CD4^+$ T cell infiltration	233,284	237
Increased Th cytokines	235,262,284,289	286
Improved corneal barrier with corticosteroids	222	290–292
Improved conjunctival goblet cells with cyclosporine	293	294,295

Table 2. 2 Comparison between desiccating stress murine model and human dry eye disease
From Stern and Pflugfelder 2017.

In addition to exposure to desiccating stress, it is important to note that some groups also treat mice with anti-muscarinic agents (such as scopolamine).[296] However, previous work from the Dana laboratory has established that exposure to desiccating stress alone is effective in inducing DED.[297] Activation of muscarinic acetylcholine receptors has been shown to upregulate intracellular calcium signalling and increase c-fos mRNA expression,[298] induce IL-2 production,[299] increase T-cell proliferation[300] and limit IFNγ synthesis.[301] In order to negate these potential confounding phenomena, I chose not administer scopolamine in the DED experiments I conducted.

2.5.1 The controlled environment chamber

A controlled environment has previously been designed and built for the Dana laboratory, that permits the regulation of airflow, humidity and temperature.[274] The chamber consists of a dry cabinet (Xdry XD1-702-02, Totech America Cooporation, Las Vegas, NV, USA) with air supply lines. These air lines connect to a compressed air source. Air flow is filtered through a membrane dryer (Drypoint M 10-47A, Beko Technologies Corp, Atlanta, GA, USA). The controlled environment chamber allows

regulation of relative humidity below 20%, a constant temperature of 21-23°C and an airflow of 15L/min. The temperature and humidity in the controlled environment chamber are continuously monitored by a probe, and the readings are documented on circular charts by a temperature humidity recorder (Supco, Allenwood, NJ, USA). Dry air is pumped directly into mouse cages (situated inside the cabinet) through four 1mm plastic tips placed in opposing walls of each cage by a linear pump (Gast Manufacturing, Benton Harbor, MI, USA). The airflow is monitored by a flowmeter. During induction of dry eye disease, mice are exposed to these conditions for 24 hours/day.[252,262]

2.5.2 Clinical assessment

Corneal fluorescein staining (CFS) was performed in order to evaluate corneal epitheliopathy following exposure to desiccating stress. 1 µl of 1% fluorescein (Sigma-Aldrich, St. Louis, MO) was administered to the lateral conjunctival sac using a micropipette. After 3 minutes, the eyes were examined by slit-lamp biomicroscopy under a cobalt blue light. The National Eye Institute (Bethesda, MD, USA) grading system was used to evaluate punctate staining, with a score of 0-3 given to each of the five areas of the cornea (i.e. total score: 3 minimum, 15 maximum) (Figure 2. 4). [302]

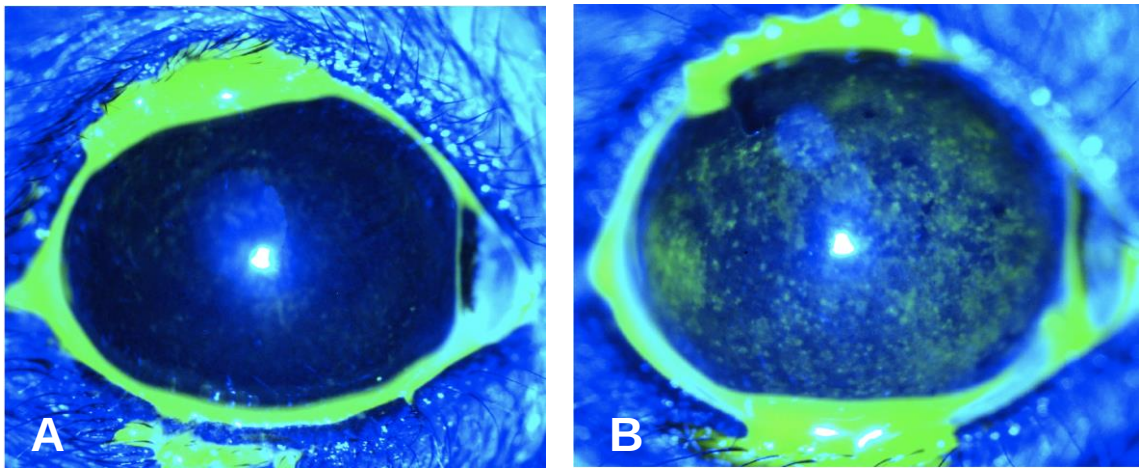

Figure 2. 4 Representative corneal fluorescein staining of ocular surface
Minimal (A) and severe (B) corneal epitheliopathy in 8-week old mice housed in the controlled environment chamber for 14 days

2.5.3 Age of mice employed in ageing studies

Female C57BL/6 mice used in these experiments were acquired from Charles River Laboratories. At time of initial exposure to desiccating stress, young mice were 6 to 8 weeks of age. Aged mice used in these studies were retired breeders, purchased at approximately 8 months of age. Subsequently, these mice were housed in the Schepens Eye Research Institute animal vivarium for an additional 4 to 6 months. Thus, aged mice were 12-14 months old at the time of initial exposure to desiccating stress.

2.6. Flow cytometry

Flow cytometry is a widely used technique for the analysis of molecules expressed both at the cell surface and cell interior. Flow cytometry permits measurement of the intensity of fluorescence produced by fluorescent-labelled antibodies that bind to proteins or other cell-associated molecules. Antibodies and matched isotype controls were purchased from Biolegend, San Diego, CA, USA.

2.6.1 Preparation of single-cell suspensions

Corneas and conjunctivae were harvested using a 2mm trephine (to mark the cornea), a 30G needle (to penetrate the anterior chamber), jewellers forceps and Vannas scissors. Collagenase digestion was used to prepare single-cell suspensions from harvested corneas and conjunctivae. Corneas and conjunctivae were digested in RPMI media (Lonza, Walkersville, MD) containing 2mg/ml DNase I (Roche, Basel, Switzerland) and 2 mg/ml collagenase type IV (Sigma-Aldrich, St. Louis, MO) for 60 minutes at 37 °C. Prior to digesting corneas, I made a series of incisions using Vannas scissors from the periphery of the corneal button toward the centre, in order to increase the surface area exposed to DNase I and collagenase. Following digestion,

samples were filtered through a 70 µm cell strainer (Corning Falcon, Thermo Fisher Scientific, Waltham, MA, USA).

Draining lymph nodes in the submandibular and cervical regions were harvested using jewellers' forceps. Thus, unilateral lymph nodes were collected for corneal transplantation and injury studies, and bilateral lymph nodes were collected for dry eye disease studies. Lymph nodes were passed through 70 µm cell strainer.

2.6.2 Surface staining

Surface staining was performed by incubating isolated cells with monoclonal antibodies or isotype matched controls for 30 minutes in the dark, on ice. Cells were subsequently washed twice using phosphate-buffered saline (PBS). Following this, stained cells were suspended in fluorescent activated cell sorting (FACS) buffer. A LSR II flow cytometer (BD Biosciences, San Jose, CA) and Summit v4.3 software (Beckman Coulter, Indianapolis, IN) were used to analyse the samples.

2.6.3 Intracellular staining

In order to evaluate the expression of IFNγ in Th1 cells and Foxp3 in $CD4^+CD25^+$ cells, intracellular staining was performed. For the intracellular quantitation of IFNγ in Th1 cells in draining lymph node samples, phorbol 12-myristate 13-acetate (PMA; 20 ng/ml; Sigma Aldrich, St Louis, MO, USA) and ionomycin (1 µg/mL; Sigma Aldrich) were used to stimulate cells for 4 hours in the presence of Golgistop (0.1 µL/100µL media; BD Biosciences, San Jose, CA, USA). Following surface staining, cells were washed twice with PBS. Cells were subsequently resuspended in Fixation/Permeabilization solution (eBioscience, Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 4°C for 60 minutes. After this, fixed and permeabilized cells were washed with Perm/Wash Buffer (eBioscience) and stained with the appropriate antibodies or matched isotype controls for 45 minutes on ice. As with

surface stained samples, a LSR II flow cytometer (BD Biosciences, San Jose, CA) and Summit v4.3 software (Beckman Coulter, Indianapolis, IN) were used to analyse the samples.

2.6.4 Gating strategies

Doublet discrimination was performed by gating simultaneously on FSC_{area} and FSC_{height}. Details of the specific antibodies and isotype controls used, as well as the gating strategies for each population of cells, are provided in their respective chapters.

2.7. Real-time polymerase chain reaction

Total RNA was isolated using a commercially available kit (RNeasy Micro Kit; Qiagen, Valencia, CA, USA). RNA was reverse transcribed into cDNA using reverse transcriptase (Superscript III; Invitrogen, Carsbad, CA, USA). Subsequently, quantitative real-time PCR was conducted in a Mastercycler RealPlex2 (Eppendorf, Hamburg, Germany) using preformulated Taqman-based probes for murine hepatocyte growth factor (*Hgf,* Mm01135184_m1) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh,* Mm99999915_gl) with Taqman Universal PCR Mastermix (Thermo Fisher Scientific, Waltham, MA, USA).

2.8. Mesenchymal stem cell purification and expansion

C57BL/6 mice were euthanized, and bone marrow was harvested from their femurs. Bone marrow cells were cultured in mouse mesenchymal stem cell (MSC) medium (Stem Cell Technologies Inc, Vancouver, Canada) using the plastic adherence method of MSC cultivation. Non-adherent cells were removed every 48 hours by changing the medium. MSCs harvested at passage 2 were used in experiments. Prior to using cultured MSCs in experiments, MSCs were characterized both phenotypically as $CD45^{-}CD34^{-}SCA1^{+}CD29^{+}$ and functionally by differentiation into adipocytes, as per the

criteria set forth by the International Society for Cellular Therapy.[190,191,199,303] Please note that the MSC purification and expansion experiments were performed primarily by my colleague Sharad Mittal PhD at Schepens Eye Research Institute.[304]

2.9. siRNA transfection

MSCs (1.5 x 10^6 cells) were plated and incubated using a 75 cm^2 culture flask for 18-24 hours. After reaching 60-70% confluency, the cells were washed and transfected with 4.8 µg of *Hgf*-specific or non-specific control siRNA using transfection reagent in siRNA transfection medium, according to the manufacturer's protocol (Santa Cruz Biotechnology, Dallas, TX, USA). After overnight incubation, the transfection medium was replaced by standard MSC growth culture medium, and the culture continued for a further 48 hours. The knockdown efficiency of siRNA after 3 and 7 days of transfection was confirmed by real-time PCR using *Hgf*-specific primers. Please note that the siRNA transfection was performed primarily by my colleague Sharad Mittal PhD at Schepens Eye Research Institute.[304]

2.10. Cell culture assays

Bone marrow-derived dendritic cells were generated by culturing bone marrow cells in the presence of granulocyte-macrophage colony-stimulating factor (20 ng/ml) for 6 days. Cultures were observed to contain >8%% $CD11c^{high}$ cells. For dendritic cell-MSC co-culture assays, dendritic cells were cultured either alone or on a monolayer of MSCs, with or without IL-1β (100 ng/ml) for 24 hours. For the *in vitro* HGF neutralization assays, co-cultures were treated with soluble HGF receptor at the doses of 1 µg/ml and 10 µg/ml. Please note that the cell culture assays were performed primarily by my colleague Sharad Mittal PhD at Schepens Eye Research Institute.[304]

2.11. Enzyme-linked immunosorbent assay

Commercially-available murine enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems; Abcam, Cambridge, MA, USA) were used to analyse protein levels of HGF in MSC culture supernatants as per the manufacturer's instructions.

2.12. Magnetic cell sorting

$CD4^+CD25^+$ regulatory T cells and $CD4^+CD25^-$ responder cells were sorted by magnetic separation using Treg isolation kits (Miltenyi Biotec, Bergisch-Gladbach, Germany) as per the manufacturer's instructions. The purity of isolated Tregs was confirmed as >90% by flow cytometry. APCs were obtained by depleting $CD90.2^+$ T cells from splenocytes using a CD90.2 isolation kit (MIltenyi Biotec).

2.13. Statistical analysis

Unpaired two-tailed Student's t-tests were used in all analysis except for the results depicted in 5.3 and 5.9, in which Mann-Whitney *U* tests were used. Significance level was set at $p < 0.05$. Corneal transplantation survival curves were constructed using Kaplan-Meier analysis, and the log-rank test was used to compare corneal graft survival between groups. Data are presented as mean ± standard error of the mean. Samples sizes were based on previous experimental studies of corneal transplantation, dry eye disease and corneal injury.[78,128,164,199,250,252,253,271]

CHAPTER 3. Corneal transplantation: the effect of the purinergic receptor antagonist oxidized adenosine triphosphate on immune-mediated allograft rejection

3.1. Introduction

3.1.1. Purinergic system and the immune response

In proposing his pattern recognition theory in 1989, Charles Janeway predicted that the innate immune system controlled activation of the adaptive immune response.[305] Janeway suggested that germline-encoded pattern recognition receptors (PRRs) on innate cells might identify nonself pathogen-associated molecular patterns (PAMPs).[305] This idea is at the core of the three signal paradigm of innate control of adaptive immunity.[306] The first signal is presentation of antigen by innate cells, with subsequent T cell receptor activation and clonal expansion of antigen-specific T cells. The second signal is provided via costimulatory molecules on APCs, the expression of which is upregulated on APCs when antigen is associated with a PAMP. Finally, activation of PRRs results in the release of innate cytokines, with this cytokine milieu promoting the differentiation of T cells into antigen-specific protective subsets. Although this model of innate control of the adaptive immune response has provided powerful mechanistic insights, there is mounting evidence that the three signal paradigm is a vast oversimplification of innate control of adaptive immunity.[306] Indeed, there is evidence that purinergic signalling may modulate the adaptive immune response in multiple ways.[307]

3.1.1.1 Temporal phases of purinergic signalling following tissue injury

Following tissue injury, purinergic signalling can be understood as occurring in three temporal phases (Figure 3. 1).[307] In the first (acute) phase, adenosine triphosphate (ATP) is released into the extracellular space either passively by necrotic cells following cellular damage, or actively by apoptotic cells and activated immune cells.[308] Accumulating to high levels, extracellular ATP has excitatory and chemotactic effects on immune cells. Extracellular ATP binds to excitatory receptors including inotropic P2XR and metabotropic P2YR subtypes, and serves to promote inflammasome

activation in dendritic cells and macrophages and amplify T cell signalling.[309] Over the ensuing hours to days, ATP is converted to adenosine diphosphate (ADP) and adenosine monophosphate (AMP) by ectonucleotidase enzymes including CD39.[307] AMP is subsequently converted to adenosine by CD73. During the second (subacute) phase, the decrease in the extracellular ratio of ATP:adenosine results in increased activation of adenosine receptors (including A2A and A2B receptors) which have an anti-inflammatory effect.[307] Adenosine signalling is halted when adenosine is converted into inosine by adenosine deaminase.[308] The third (chronic phase) of purinergic signalling occurs in the days to weeks following tissue injury, corresponds to a low extracellular ratio of ATP:adenosine, and is associated with wound healing processes and tissue remodelling.[307]

Figure redacted for copyright reasons

Figure 3. 1 Three phases of purinergic signaling following tissue injury

Tissue injury results in the release of high levels of adenosine triphosphate into the extracellular environment. ATP is subsequently dephosphorylated to adenosine diphosphate, adenosine monophosphate and adenosine by ectonucleotidase enzymes including CD39 and CD73. The various effects of the decreasing ATP:adenosine ratio can be understood as occurring in three temporal phases, as shown. From Cekic and Linden 2016

3.1.1.2 Expression of purinergic receptors by immune cells

ATP and uridine-5'-triphosphate (UTP), as well other nucleotides, are released via pannexins, connexins, maxichannels and P2X7R pores during apoptosis or cellular

stress.[310,311] These nucleotides function to activate platelets, attract phagocytes and promote local vasodilation.[307] Although the expression of purinergic receptors by immune cells has not yet been fully deciphered, there is evidence that the majority of immune cells express a combination of P2X and P2Y receptors (Table 3. 1).[307,309]

Table redacted for copyright reasons

Table 3. 1 Expression of purinergic receptors by immune cells
nd: not determined. Adapted from Junger 2014

The effect of binding of ATP and other nucleotides to P2 purinergic receptors is predominantly excitatory. Inotropic P2X receptors bind ATP and permit the intracellular passage of Na^+, K^+ and Ca^{2+}. It is noteworthy that P2X7Rs have a relatively low affinity for ATP, and thus are activated only in highly inflamed environments.[307] Furthermore, P2X7R has been implicated in T cell activation and function,[312,313] and inhibition of

P2X7R has been shown to promote redirection of the immune response from a pro-inflammatory Th1/Th17 response to more tolerogenic condition.[309] G-coupled metabotropic P2YRs also promote immune cell activation by permitting calcium mobilization and limiting the accumulation of anti-inflammatory cyclic AMP.[307]

In contrast to the P2 purinergic receptors, adenosine signalling generally has anti-inflammatory effects on immune cells. Indeed, deficiency of A2a adenosine receptors has been shown to render mice susceptible to excessive inflammation and tissue damage in response to innocuous stimuli.[314] There are four subtypes of adenosine receptor (Table 3. 1), and of these, the expression of A2a and A2b receptors (G protein-coupled) is increased following immune cell activation. In response to adenosine ligation, these receptors serve to limit inflammation, with increased generation of cyclic AMP and activation of protein kinase A.[307]

3.1.2. Purinergic regulation of the alloimmune response

As our understanding of the expression of purinergic receptors and their immunomodulatory activities has developed, studies have been conducted investigating how the purinergic system influences the alloimmune response. In particular, three studies have been published by the Fiorina group have investigated the effect of the purinergic receptor inhibitor oxidized ATP (oATP) in heart[315], lung[316] and islet[317] transplantation.

In their cardiac transplantation studies, Vergani and colleagues report that expression of P2X7R is specifically increased in graft-infiltrating lymphocytes in humans and mice.[315] The authors demonstrate 80% survival of fully-mismatched murine allografts following treatment with an ATP antagonist, oxidized ATP. The investigators also report decreased T cell activation and Th1/Th17 differentiation. The effect of oATP on APC frequencies and function was not assessed. No difference was observed in Treg

frequencies between groups at day 7 post-transplantation, which approximates the time at which all untreated grafts had been rejected but did observe increased Treg frequencies at day 100 post-transplantation. Interestingly, frequencies of Tregs in P2X7R-deficient mice were significantly increased relative to wild-type controls.[315]

Using a murine model of lung transplantation, Liu and colleagues treated graft recipients with oATP after the onset of transplant rejection to evaluate its capacity to rescue lung grafts.[316] The investigators found that oATP prolonged allograft survival, with decreased frequencies of infiltrating inflammatory cells and improved lung function. The authors observed reduced frequencies of $CD4^+$ T cells in oATP-treated grafts. In contrast with the Vergani cardiac study[315], a transient short-term increase in Treg frequencies both in the lungs and spleen was observed (i.e. at day 15 post-transplantation), with no difference demonstrated at two later timepoints.[316] The effect of oATP on antigen-presenting cells was not evaluated. In another study, Vergani and colleagues confirmed that in oATP reduced frequencies of Th1/Th17 cells and promotes allograft survival in a murine model of islet transplantation.[317] Notably, the authors did not observe a difference in peripheral frequencies of either $CD44^{high}CD62^{low}$ $CD4^+$ effector T cells or Tregs at 14 days post-transplantation.[317]

3.1.3. Research questions

Taken together, the studies described in Section 3.1.2 provide strong evidence that antagonizing purinergic receptors with oATP effectively reduces the severity of the alloimmune response and prolongs graft survival. Yet several unanswered questions remain:

- Firstly, what is the effect of oATP on APCs in the setting of alloimmunity? Relatedly, does oATP modulate effector T cell function directly, or via APCs?
- Secondly, what is the effect of oATP on Treg frequencies and function in allotransplantation? Although the studies from the Fiorina group have

evaluated Treg frequencies, there is a lack of consistency between the reports.[315–317] Moreover, outside of the transplantation literature, there is evidence of oATP mediating its therapeutic effects through the expansion and augmented function of Tregs.[318,319]

- Finally, no previous studies employing oATP in corneal transplantation have been reported, and the effect of oATP on the alloimmune response in this uniquely immunologically quiescent environment is not known.

To address these gaps in our current knowledge, I performed a series of experiments.

3.2. The effect of purinergic blockade on corneal infiltration of leukocytes and antigen-presenting cell maturation

3.2.1. Research question

What is the effect of inhibiting purinergic signalling with oxidized ATP on corneal infiltration of $CD45^+$ leukocytes and APC maturation?

3.2.2. Experimental design

The mouse model of corneal transplantation was used in this experiment, as described in Chapter 2. Immediately following transplantation, the first dose of 250 µg oATP (Sigma Aldrich, St. Louis, MO) diluted 100 µl sterile saline was administered via intraperitoneal injection. Animals received one injection daily for a total of fourteen days, as depicted (Figure 3. 2). The duration of oATP treatment was based on previous studies of this agent in studies of heart, lung and islet transplantation.[315–317] Control animals received daily intraperitoneal injections of 100 µl sterile saline.

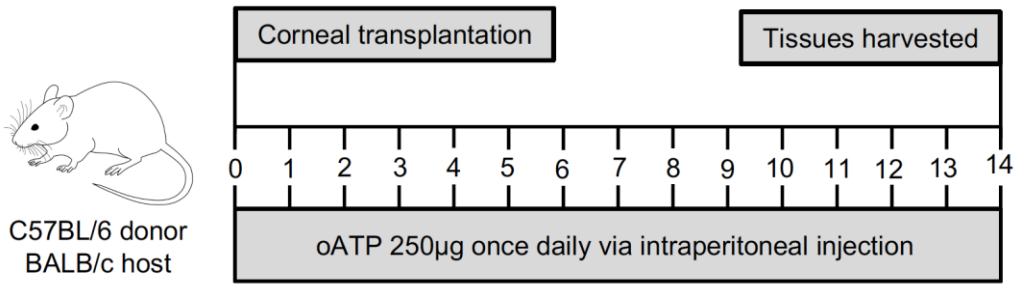

Figure 3. 2 Experiment schematic

Tissues were harvested at day 14 following transplantation, and single cell suspensions were prepared, as described in Chapter 2. Day 14 is a standard time point for immune analysis of tissues following corneal transplantation, as it provides adequate time for allosensitization and the trafficking of immune cells from draining lymph nodes to corneal tissue, and occurs prior to the onset of higher rejection rates at week 3.[155,261,320] The kinetics of this phenomenon may be related to the kinetics of

corneal angiogenesis and lymphangiogenesis following suture placement, which has been shown to peak at day 14.[321]

Flow cytometric analyses were used in this experiment, as described in Chapter 2. Fluorescently-conjugated antibodies against CD45, CD11b and MHCII as well as their matched isotype controls were used in this experiment.

3.2.3. Results

Flow cytometric data demonstrated an increase in the infiltration of $CD45^+$ inflammatory cells into the cornea in saline-treated animals, an effect that was substantially decreased in the oATP-treated group (Figure 3. 3). A similar pattern was observed when the frequencies of $MHCII^+CD11b^+$ mature APCs were examined in the cornea, with an almost 3-fold reduction in mature APCs observed in oATP-treated mice compared to saline-treated control mice (Figure 3. 4). The frequencies of $MHCII^+CD11b^+$ mature APCs were also examined in the draining lymph nodes, where a comparable fold decrease in the frequencies of mature APCs was evident following treatment with oATP (Figure 3. 5). Having observed this decrease in frequencies of mature APCs in both the cornea and draining lymph nodes following treatment with oATP, I next sought to investigate whether administration of oATP suppressed the generation of effector T cells.

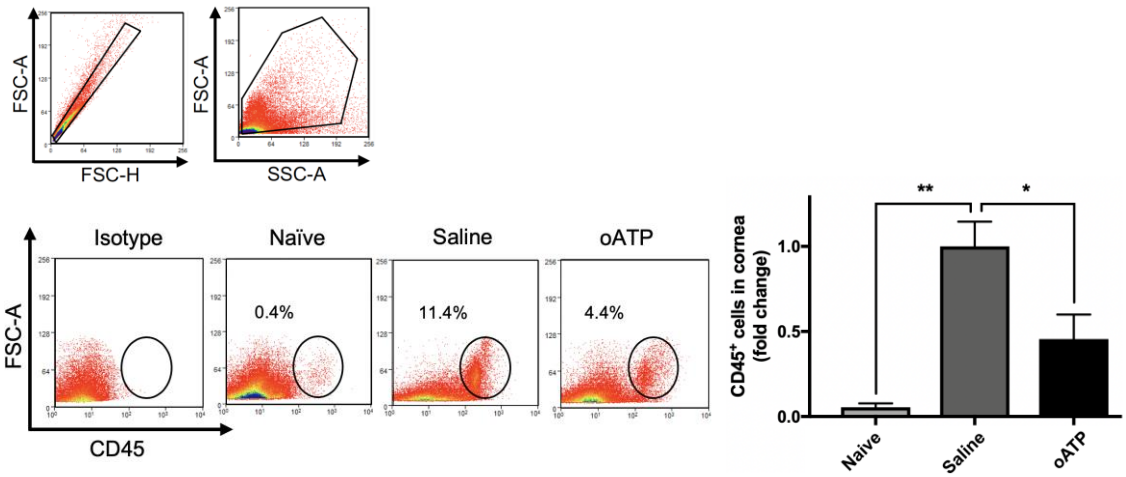

Figure 3. 3 Frequencies of CD45⁺ cells in the cornea

Representative flow cytometry dot plots showing the gating strategy for (upper), and frequencies of (lower left), CD45⁺ inflammatory cells in the corneas of oATP-treated mice, compared to saline-treated and naïve mice. Tissues were collected at 14 days post-transplantation. 2-3 corneas were pooled per sample. n=5-7/group. Bar chart (right) shows fold change data pooled from multiple experiments. Data are depicted as mean +/- SEM. *p<0.05; **p<0.01.

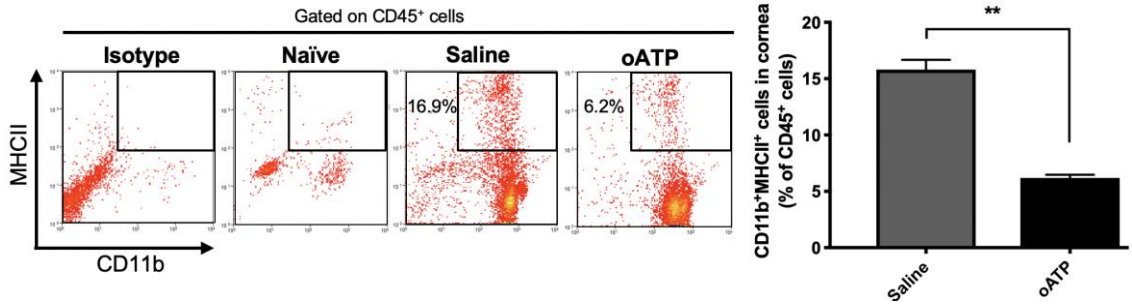

Figure 3. 4 Frequencies of CD11b⁺MHCII⁺ cells in the cornea

Representative flow cytometry dot plots (left) and bar chart (right) showing the frequencies of CD11b⁺MHCII⁺ cells in the cornea at 14 days post-transplantation. Data shown are gated on CD45⁺ cells. 2-3 corneas were pooled per sample. n=5-7 per group. Data are depicted as mean +/- SEM. **p<0.01.

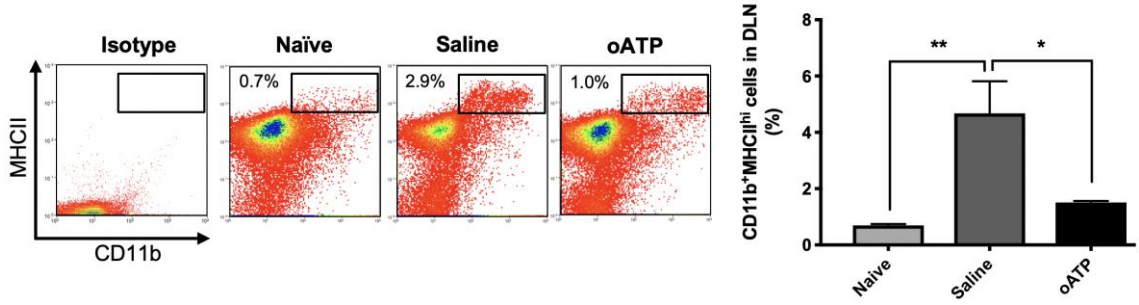

Figure 3. 5 Frequencies of CD11b⁺MHCII⁺ cells in the draining lymph nodes

Representative flow cytometry dot plots (left) and bar chart (right) showing the frequencies of CD11b⁺MHCII⁺ cells in the draining lymph nodes of naïve, saline-treated and oATP-treated corneal transplant recipients at 14 days post-transplantation. n=5-7 per group. Data are depicted as mean +/- SEM. *p<0.05; **p<0.01.

3.3. The effect of purinergic blockade on effector T cell generation

3.3.1. Research question

What is the effect of inhibiting purinergic signalling with oxidized ATP on Th1 frequencies in the draining lymph nodes and the corneal infiltration of $CD4^+$ T cells?

3.3.2. Experimental design

The mouse model of corneal transplantation was used, as described in Chapter 2. oATP was administered as detailed in Section 3.2.2. Tissues were harvested and flow cytometry was performed as described in Section 3.2.2. Fluorescently-conjugated antibodies against CD4 and IFNγ, as well as their matched isotype controls were used in this experiment.

3.3.3. Results

As part of the alloimmune response, the inflammatory milieu at the cornea promotes the expression of MHCII and co-stimulatory molecules such as CD80 and CD86 by APCs.[79] Following maturation, these APCs migrate via lymphatic vessels to the draining lymph nodes, where they present alloantigens and prime naïve T cells, driving the expansion and differentiation of $CD4^+IFN\gamma^+$ Th1 cells.[79] Here, I investigated whether the reduced frequencies of mature APCs observed in the draining lymph nodes of mice treated with oATP was associated with reduced frequencies of Th1 cells. As anticipated based on previous publications,[165,322] I observed a substantial increase in frequencies of $CD4^+IFN\gamma^+$ Th1 cells in saline-treated mice following transplantation, relative to naïve mice (Figure 3. 6). This expansion of Th1 cells was abrogated in the oATP-treated mice. I also evaluated corneal infiltration of $CD4^+$ T cells, with these data exhibiting a similar pattern of increased frequencies in the saline-treated recipients relative to naïve mice, and a significant reduction in $CD4^+$ T cell infiltration in the oATP-treated hosts compared to saline-treated hosts (Figure 3. 7).

Having observed that administration of oATP suppressed the generation of Th1 cells in lymphoid tissues, and also limited the infiltration of $CD4^+$ cells into donor corneal tissue, I next sought to establish whether treatment with oATP also modulated the regulatory arm of the immune response.

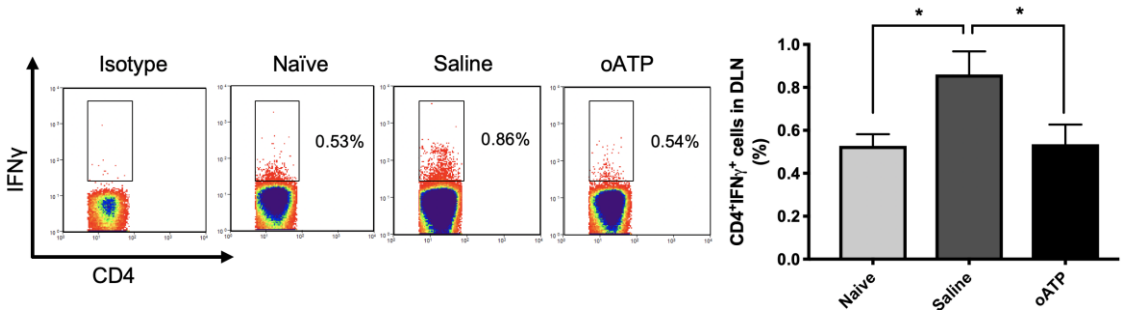

Figure 3. 6 Frequencies of $CD4^+IFN\gamma^+$ Th1 cells in the draining lymph nodes

Representative flow cytometry dot plots (left) and bar chart (right) showing the frequencies of $CD4^+IFN\gamma^+$ cells in the draining lymph nodes of naïve, saline-treated and oATP-treated corneal transplant recipients at 14 days post-transplantation. n=5-7 per group. Data are depicted as mean +/- SEM. *p<0.05.

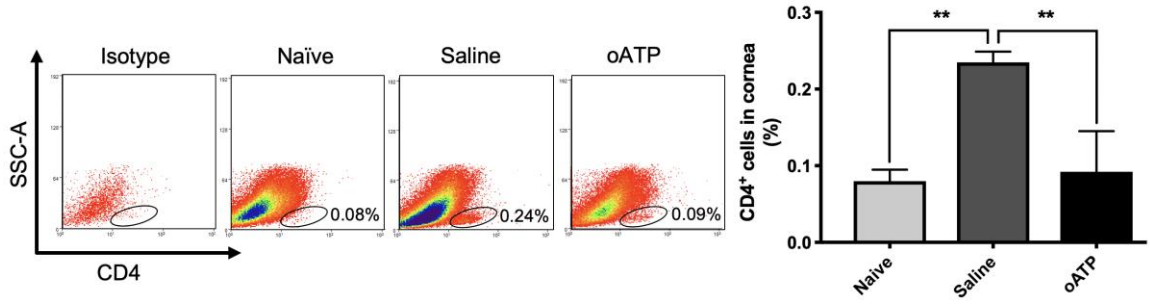

Figure 3. 7 Frequencies of graft-infiltrating $CD4^+$ cells

Representative flow cytometry dot plots (left) and bar chart (right) showing the frequencies of $CD4^+$ cells in the corneas of naïve, saline-treated and oATP-treated corneal transplant recipients at 14 days post-transplantation. 2-3 corneas were pooled per sample. n=5-7 per group. Data are depicted as mean +/- SEM. **p<0.01.

3.4. The effect of purinergic blockade on regulatory T cell generation

3.4.1. Research question

What is the effect of inhibiting purinergic signalling with oxidized ATP on Treg frequencies in the draining lymph nodes and cornea?

3.4.2. Experimental design

The mouse model of corneal transplantation was used, as described in Chapter 2. oATP was administered as detailed in Section 3.2.2. Tissues were harvested and flow cytometry was performed as described in Section 3.2.2. Fluorescently-conjugated antibodies against CD4 and Foxp3, as well as their matched isotype controls were used in this experiment.

3.4.3. Results

Analysis of the frequencies of $CD4^+Foxp3^+$ cells in the draining lymph nodes of naïve mice, saline-treated allograft hosts and oATP-treated allograft hosts did not reveal significant differences between groups (Figure 3. 8). Furthermore, no difference in the expression of Foxp3 in saline- vs. oATP-treated hosts was observed (mean fluorescence intensity equal to 43 and 40 respectively; $p = 0.055$). Similar findings were observed in corneal single cell suspensions (Figure 3. 9), with no significant differences in the frequencies of $CD4^+Foxp3^+$ cells or Foxp3 expression between saline- and oATP-treated groups (Foxp3 mean fluorescence intensity equal to 28 and 27 respectively; $p = 0.785$).

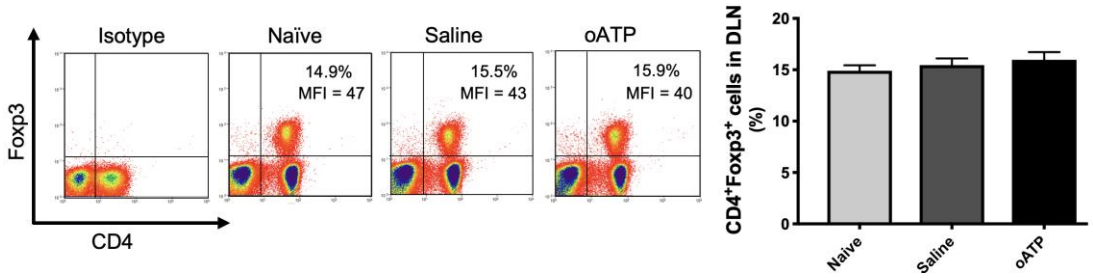

Figure 3. 8 Frequencies of CD4$^+$Foxp3$^+$ Tregs in the draining lymph nodes

Representative flow cytometry dot plots (left) and bar chart (right) showing the frequencies of CD4$^+$Foxp3$^+$ cells in the draining lymph nodes of naïve, saline-treated and oATP-treated corneal transplant recipients at 14 days post-transplantation. n=5-7 per group. Data are depicted as mean +/- SEM.

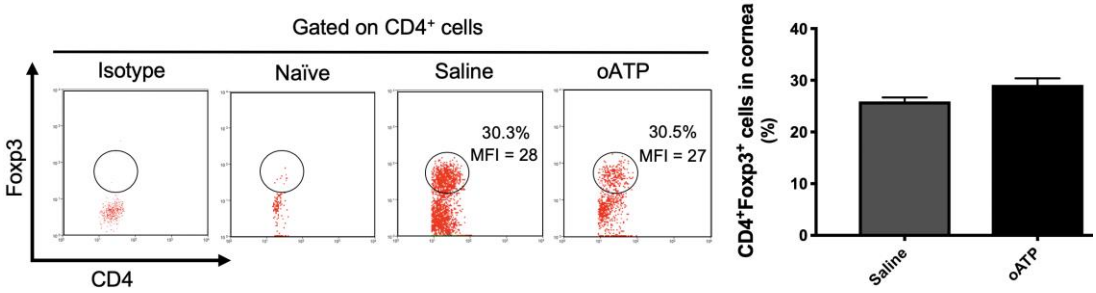

Figure 3. 9 Frequencies of corneal CD4$^+$Foxp3$^+$ Tregs

Representative flow cytometry dot plots (left) and bar chart (right) showing the frequencies of CD4$^+$Foxp3$^+$ cells in the corneas of naïve, saline-treated and oATP-treated corneal transplant recipients at 14 days post-transplantation. Data shown is gated on CD4$^+$ cells. 2-3 corneas were pooled per sample. n=5-7 per group. Data are depicted as mean +/- SEM.

3.5. The effect of purinergic blockade on regulatory T cell suppressive function

3.5.1. Research question

Having observed that oATP administration did not significantly modulate Treg frequencies either locally at the cornea, or in the lymphoid tissue, I next asked the question: what is the effect of inhibiting purinergic signalling with oxidized ATP on the capacity of Tregs to suppress $CD4^+CD25^-$ T cell proliferation?

3.5.2. Experimental design

$CD4^+CD25^+$ Tregs were isolated from the spleens and lymph nodes of BALB/c mice using magnetic sorting kits (Miltenyi Biotec), as described in Chapter 2. The naïve T cells ($1x10^5$) were labelled with carboxyfluorescein succinimidyl ester (CFSE) (5 μM; Invitrogen) and co-cultured with oATP (500 μM; Sigma Aldrich)-treated $CD4^+CD25^+$ Tregs ($5x10^4$), T cell-depleted syngeneic splenocytes ($1x10^5$) and 1 μg/ml anti-CD3 antibody for 3 days in the presence of exogenous ATP (100nM; Tocris). T cells cultured alone served as controls.

3.5.3. Results

In vitro functional assays of Treg suppression of T cell proliferation were performed to assess the capacity of oATP-treated vs. untreated Tregs to suppress T cell proliferation. oATP-treatment of Tregs did not result in a substantial reduction in T cell proliferation as compared to untreated Tregs (Figure 3. 10).

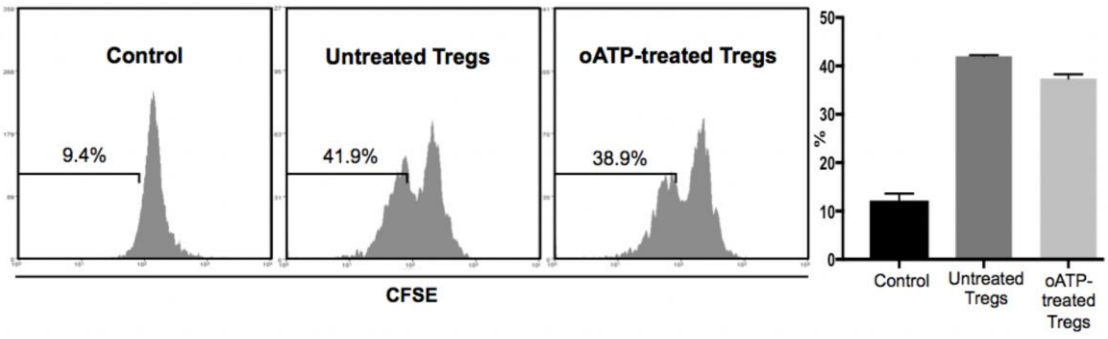

Figure 3. 10 oATP treatment does not substantially modulate Treg suppressive function

Representative histograms (left) and bar chart (right) show comparable dilution of CFSE in proliferating T cells cultured with oATP-treated or PBS-treated Tregs. Data are depicted as mean +/- SEM.

3.6. The direct effect of oxidized ATP on T cells and antigen presenting cells

3.6.1. Research question

Having determined that administration of oATP limited frequencies of mature APCs both at the cornea and draining lymph nodes, as well as frequencies of graft-attacking Th1 cells, I next sought to establish whether in addition to indirect suppression via APCs, oATP also modulated Th1 frequencies directly. Specifically, I sought to determine the direct effect of oATP on T cells and APCs using mixed lymphocyte reactions.

3.6.2. Experimental design

T cells ($CD90.2^+$) from BALB/c mice and APCs ($CD90.2^-$) from C57BL/6 mice were purified from spleens and lymph nodes by magnetic sorting, as described in Chapter 2. Isolated T cells were labelled with CFSE. Subsequently, oATP-treated or PBS-treated T cells were co-cultured with oATP-treated or PBS-treated APCs in the media containing exogenous ATP for 5 days.

3.6.3. Results

In vitro assays were conducted to evaluate the direct effects of oATP on T cells and on the maturation of APCs (Figure 3. 11). These data show that, in addition to its APC-mediated immunoregulatory activity, oATP has a direct effect on alloreactive T cells. Notably, treatment of T cells with oATP resulted in a 49% reduction in dilution of CFSE-labeled cells, compared with a 95% reduction when APCs were treated with oATP. These data demonstrate a direct effect of oATP on T cell function, but also indicate that modulation of APCs is the dominant of these two phenomena. Indeed, when APCs were treated with oATP, the levels of dilution of CFSE-labeled cells were similar regardless of whether or not T cells were treated with oATP.

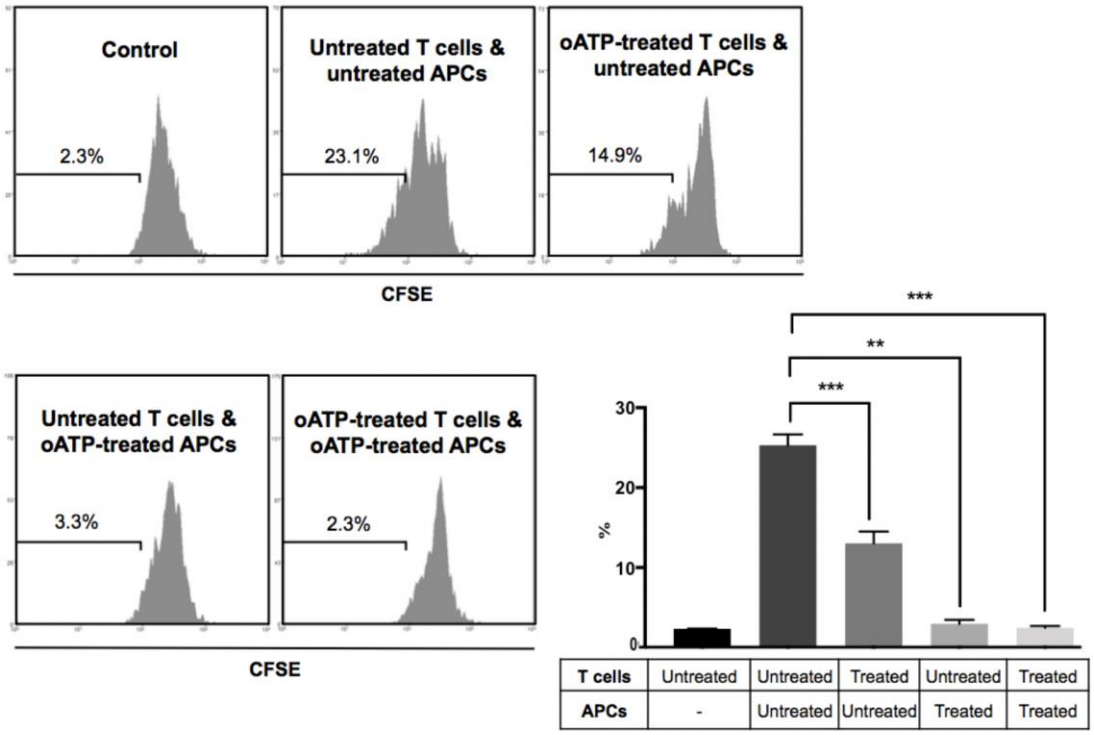

Figure 3. 11 oATP suppresses antigen-presenting cells and T cells in mixed lymphocyte reactions

Histograms (top and left) and bar chart (bottom right) depicting the proliferation of CFSE-labeled T cells in mixed lymphocyte reaction cultures following indicated treatments. Data are depicted as mean +/- SEM. *p<0.05.

3.7. The effect of purinergic blockade on corneal allograft opacity and survival

3.7.1. Research question

Having evaluated the effects of oxidized ATP on the effector and regulatory arms of the alloimmune response, I next asked the question, what is the effect of blocking purinergic signalling with oxidized ATP on graft opacity and corneal transplant survival?

3.7.2. Experimental design

The mouse model of corneal transplantation was used, as described in Chapter 2. oATP was administered as detailed in Section 3.2.2. Graft opacity was assessed by slit lamp microscopy as described in Chapter 2. Graft opacity was evaluated weekly for eight weeks (as shown in **Error! Reference source not found.**).

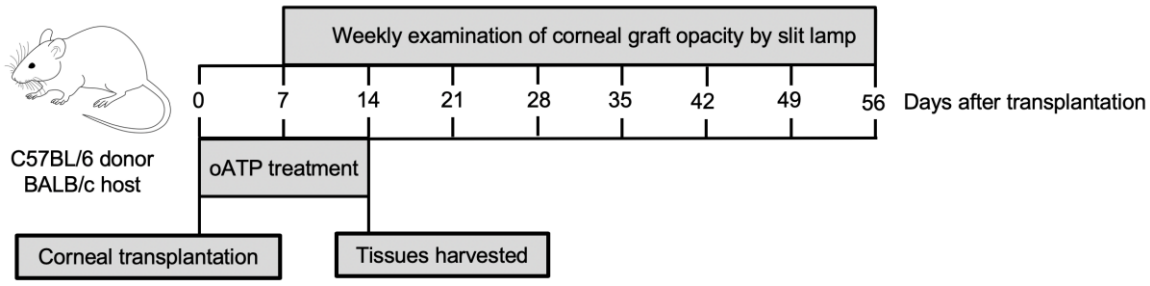

Figure 3. 12 Experiment schematic

3.7.3. Results

From postoperative week 4 onwards, the oATP-treated allograft recipients demonstrated significantly lower graft opacity scores relative to the saline-treated allograft recipients (). Similarly, significantly enhanced graft survival was observed in the oATP-treated hosts compared to the saline-treated hosts ().

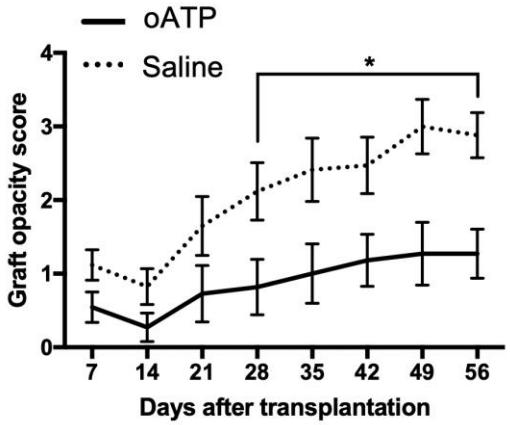

Figure 3. 13 Graft opacity scores

Graft opacity was in the respective groups was assessed by slit-lamp biomicroscopy on a weekly basis accordingly to an established scoring system. n=8 per group. Data are depicted as mean +/- SEM. *p<0.05.

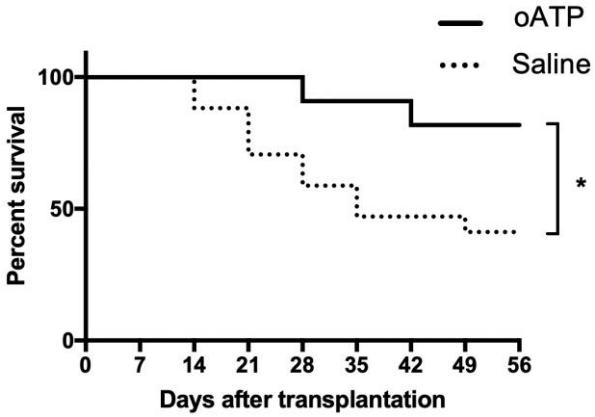

Figure 3. 14 Kaplan-Meier survival curve

Logrank test demonstrates significantly enhanced survival in the oATP-treated recipients relative to saline-treated recipients (p = 0.032). n=8 per group. Data are depicted as mean +/- SEM. *p<0.05.

3.8. Discussion

The activation of purinergic P2X and P2Y receptors by extracellular ATP augments the inflammatory response.[307,309] In these experiments, the immunomodulatory capacity of oATP, an irreversible antagonist of ATP, has been investigated in the setting of corneal allotransplantation. The data resulting from these experiments indicate that following corneal grafting, treatment with oATP suppresses corneal infiltration of leucocytes and limits APC maturation, reduces frequencies of Th1 cells in the draining lymph nodes, decreases graft infiltration by $CD4^+$ T cells and does not significantly increase either Treg frequency or function. These data also demonstrate that oATP increases corneal allograft survival.

In previous studies investigating the effect of oATP on allograft survival (in heart[315], lung[316] and islet[317] transplantation), oATP-mediated suppression of APC maturation has not been reported. Yet APCs are known to express certain purinergic receptors, including P2X7R, that promote the expression of MHCII and other co-stimulatory molecules upon activation.[307] Our data clearly indicate that inhibition of purinergic signalling with oATP leads to reduced frequencies of mature APCs both at the cornea and draining lymph nodes. By suppressing the maturation of APCs in corneal transplantation, oATP limits the differentiation and clonal expansion of graft-attacking Th1 cells.

The presented flow cytometric data indicate that oATP abrogates the increased frequencies of $CD4^+IFN\gamma^+$ Th1 cells observed in the draining lymph nodes of saline-treated mice at 14 days post-transplantation. Moreover, the corneal infiltration of $CD4^+$ T cells is similarly reduced by treatment with oATP. These data are consistent with findings from other groups demonstrating that oATP limits the Th1 alloimmune response.[315–317] Indeed, in a mouse model of heart transplantation, Vergani and colleagues report that oATP administration promoted allograft survival, and observed

decreased frequencies of IFNγ-producing cells in spleens from oATP-treated animals relative to untreated controls.[315] Intriguingly, this difference was preserved at 100 days post-transplantation.[315] The same group has reported similar observations using a mouse model of islet transplantation.[317] Another interesting point to note that is related to these studies is that short-term systemic administration of oATP was shown to increase long-term graft survival.[315–317] This is similar to the graft survival experiments that I conducted, in which a two-week treatment regimen was effective in promoting graft survival at 8 weeks post-transplantation. Collectively, these observations suggest that by modulating the acute phase of the alloimmune response, oATP has long-lasting effects on transplant survival. It is noteworthy that in other studies employing our murine model of corneal transplantation, early interventions to suppress alloimmunity (i.e. treatments limited to the first 14 days post-transplantation) have also been shown to be effective in promoting long-term graft survival.[164,168,323] This is similar to findings in other experimental models of transplantation.[324–326] Indeed, the short-term use of immunosuppressives following grafting (i.e. induction therapy) is common in clinical practice, particularly in renal transplantation.[327,328]

Tregs play an essential role in suppressing the alloimmune response in corneal transplantation.[42,155] Studies outside of the transplantation literature have suggested that antagonizing purinergic signalling (in particular the P2X7 receptor) expands Treg populations and upregulates function.[318,319] However, I observed no significant difference in $CD4^{+}Foxp3^{+}$ Treg frequencies at the draining lymph nodes or at the cornea in oATP-treated vs. saline-treated hosts. Levels of Foxp3 in Tregs have been shown to be indicative of their functional status in transplantation.[155] Yet I did not observe a significant difference in Foxp3 expression (mean fluorescence intensity) between oATP-treated and saline-treated recipients. Finally, when the suppressive function of Tregs was assessed *in vitro* using CFSE-labelling, both untreated Tregs and oATP-treated Tregs reduced T cell proliferation to a similar degree.

There are important limitations to the work presented in this chapter. Firstly, a comprehensive survey of cellular and molecular mediators of inflammation modulated by oATP was not conducted. It is plausible that the prolonged transplant survival observed in the oATP treatment group is due oATP-mediated suppression of other immune cells. Yet previous work has established that in the mouse model of corneal transplantation used in these experiments, the activation of $CD11b^{+}MHCII^{+}$ cells and the ensuing corneal infiltration of $CD4^{+}$ effector T cells plays a primary role in driving immune rejection.[97,99,134,137,162,268,322,329] A second limitation is that kinetic analysis of immune cell frequencies were not conducted, and may have captured additional immunoregulatory effects of oATP. However, previous work has established that day 14 post-transplantation is a particularly informative time point to evaluate the alloimmune response in this model, prior to the onset of higher rates of graft rejection.[162,330]

In summary, the data presented in this chapter indicate that treatment with oATP significantly decreases corneal graft opacity and increases graft survival, relative to saline-treated transplant recipients. Indeed, the data demonstrate that oATP suppresses the effector arm of the alloimmune response, with evidence of decreased APC maturation and corneal infiltration of inflammatory cells in oATP-treated mice. These findings suggest that targeting purinergic signalling may be a feasible therapeutic strategy to reduce immune rejection of allografts.

CHAPTER 4. Corneal transplantation: the effect of the mesenchymal stem cell-derived hepatocyte growth factor on corneal alloimmunity

4.1. Introduction

In Chapter 3, I investigated how blockade of purinergic signalling using oxidized ATP regulates the antigen recognition phase of the adaptive immune response using a murine model of corneal transplantation. In the experiments described in this chapter, I continued to employ this model to study how this critical phase of the alloimmune response can be regulated. Specifically, I investigated the capacity of mesenchymal stem cell (MSC)-derived hepatocyte growth factor (HGF) to suppress the alloimmune response following corneal transplantation and promote corneal graft survival. An introduction to the contact-dependent and soluble factor-mediated immunomodulatory properties of MSCs, as well as their capacity to home to sites of inflammation, is provided in Chapter 1.

MSC-derived HGF has been shown to suppress inflammation and promote epithelial repair following corneal injury has been evaluated in a study conducted by Omoto and colleagues.[200] *In vitro,* HGF was observed to inhibit the activation of $CD11b^+$ immune cells and reduce their expression of the inflammatory cytokines IL-1β and TNF-α. *In vivo*, topical application of HGF following injury promoted epithelial cell proliferation, as determined by quantification of $Ki\text{-}67^+$ cells analysed by immunohistochemistry and mRNA expression of *p63*.[200] Furthermore, topical administration of HGF suppressed inflammation following cornea injury, with reduced $CD45^+$ cells detected in the cornea by immunohistochemistry and decreased mRNA expression of IL-1β and TNF-α as compared to albumin-treated controls.[200]

4.1.4. Research questions

To date, the studies described in the introductory Section 1.5 provide evidence for the immunomodulatory and anti-fibrotic functions of HGF following corneal injury.[199,200] Yet the capacity of HGF to limit the alloimmune response following corneal transplantation, and promote corneal graft survival, is not known. Furthermore, although it has been

shown that systemic administration of corneal MSCs prolongs corneal allograft survival,[190,192–194] the immunomodulatory mechanisms of MSCs have not been clearly delineated. This chapter describes a series of experiments that were performed to answer the following questions:

- Is the capacity of MSCs to promote corneal transplant survival dependent on their expression of HGF?
- What is the effect of topical application of HGF on the induction of alloimmunity in graft recipients? Specifically, what is the effect of HGF on APC maturation and the generation of Th1 cells in the setting of corneal transplantation? Does treatment with HGF limit immune cell infiltration of the grafts?
- Is HGF alone (independently of MSCs) effective in promoting graft survival?

4.2 The effect of mesenchymal stem cell-derived hepatocyte growth factor on the maturation of antigen presenting cells

4.2.1 Research question

What is the *in vitro* effect of MSC-derived HGF on APC maturation?

4.2.2 Experimental design

MSCs were purified from bone marrow and expanded *in vitro* as described in Chapter 2. Phenotypic characterization of MSCs was performed using flow cytometry, as described in Chapter 2. Please note that the MSC purification, expansion and phenotypic characterization was performed primarily by my colleague Sharad Mittal PhD at Schepens Eye Research Institute.[304] Fluorescently-conjugated antibodies against CD45, CD34, CD29, CD11c, MHCII and CD80 as well as their matched isotype controls were used in this experiment. Commercially-available ELISA kits were used to analyse protein levels of HGF in MSC culture supernatants, as per the manufacturer's instructions.

Dendritic cells were differentiated from bone marrow cells harvested from C57BL/6 mice, as described in Chapter 2. Bone marrow-derived dendritic cells (DCs) were cultured alone or on a monolayer of MSCs either with or without IL1β (100ng/ml) for 24 hours. In the HGF neutralization assays, co-cultures were treated with soluble HGF receptor (sHGFR) at concentrations of either 1 µg/ml or 100 µg/ml. Please note that co-culture and HGF neutralization assays were performed primarily by my colleague Sharad Mittal PhD at Schepens Eye Research Institute.[304] Following this, flow cytometry was performed to evaluate expression of MHCII and CD80 by $CD11c^+$ cells.

4.2.3 Results

In order to determine whether MSCs limit the activation and maturation of APCs, we first conducted experiments in which $CD11c^+$ DCs were co-cultured with MSCs, and the expression of MHCII and CD80 was assessed using flow cytometry. In view of the limited number of dendritic cells and MSCs at the ocular surface, bone marrow was harvested from murine femurs for the isolation and generation of MSCs and $CD11c^+$ DCs. Flow cytometry was used to confirm the positive expression of the mesenchymal cell marker CD29 by MSCs, as well as the negative expression of the hematopoietic cell markers CD45 and CD34 (Figure 4. 1). In response to stimulation with the inflammatory cytokine IL1β, MSCs demonstrated increased expression of HGF ($p=0.008$; Figure 4. 2).

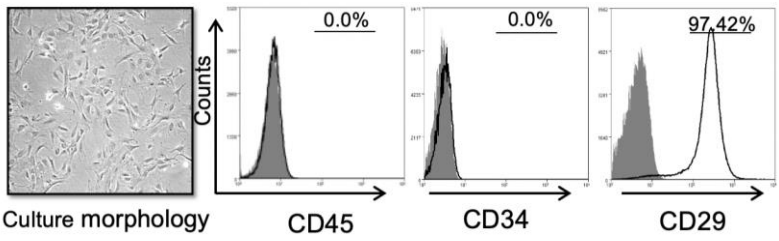

Figure 4. 1 Culture and characterization of stromal cells

MSCs were purified from bone marrow and expanded *in vitro* using MSC-conditioned culture media by the plastic adherence method. The morphology of expanded MSCs at passage two is depicted in a representative microscopic image (left). Flow cytometry was used to confirm the absence of expression of the hematopoietic cell markers CD45 and CD34, as well as the positive expression of the mesenchymal stem cell marker CD29 (right).

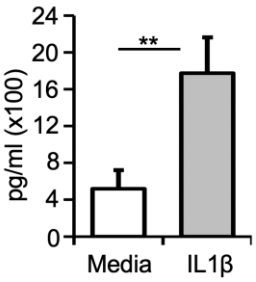

Figure 4. 2 HGF protein levels in the supernatants of MSCs cultured in media or stimulated with IL1β for 24 hours using ELISA

Data are depicted as mean +/- SD. **p<0.01

Next, DCs were stimulated with IL1β, in the presence or absence of MSCs, for 24 hours. Low expression of MHCII and CD80 was observed in unstimulated DCs, but

following stimulation with IL1β their expression increased substantially ($p<0.001$ for both MHCII and CD80; Figure 4. 3 and Figure 4. 4). Of note, this heightened expression was significantly reduced by addition of MSCs to the culture system ($p=0.003$ for MHCII, $p=0.006$ for CD80). To evaluate the possibility that this effect was mediated by HGF, we neutralized HGF by adding soluble HGF receptor (sHGFR) to the co-culture system at two different concentrations of sHGFR; 1 μg/ml and 10 μg/ml. Our results indicate that neutralization of HGF reduces MSC-mediated inhibition of IL1β-induced DC maturation (Figure 4. 3 and Figure 4. 4). Indeed, this observation was confirmed at both concentrations of sHGFR, with a dose-dependent effect evident [1 μg/ml (MHCII, $p=0.021$; CD80, not significant) and 10 μg/ml (MHCII, $p=0.014$; CD80, $p=0.015$)]. These results indicate that MSCs suppress DC maturation by the secretion of HGF.

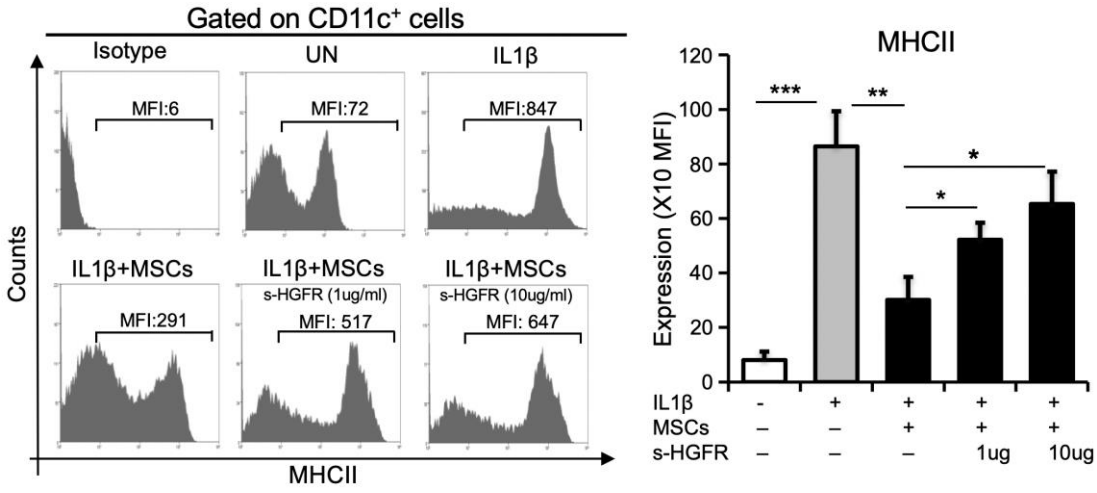

Figure 4. 3 Expression of MHCII by CD11c$^+$ cells co-cultured with MSCs with or without IL1β in the presence or absence of soluble HGF receptor

Representative FACS plots (left) and summary bar charts (right) showing the expression of MHCII on CD11c$^+$ cells co-cultured with MSCs with or without IL1β (100 ng/ml) in the presence or absence of soluble HGF receptor (1 μg/ml or 10 μg/ml, as indicated). Data are depicted as mean +/- SD. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

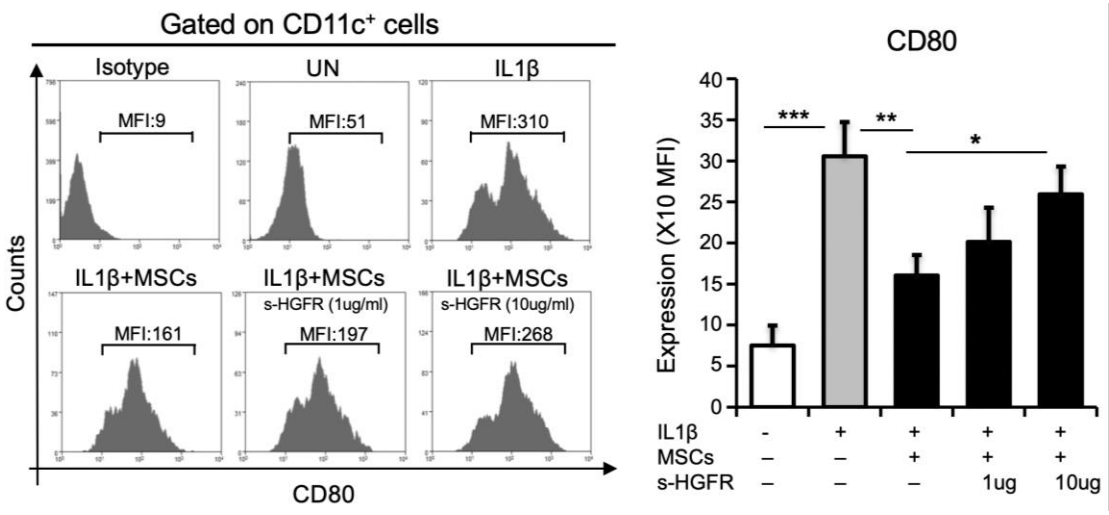

Figure 4. 4 Expression of CD80 by CD11c⁺ cells co-cultured with MSCs with or without IL1β in the presence or absence of soluble HGF receptor

Representative FACS plots (left) and summary bar charts (right) showing the expression of CD80 on CD11c⁺ cells co-cultured with MSCs with or without IL1β (100 ng/ml) in the presence or absence of soluble HGF receptor (1 μg/ml or 10 μg/ml, as indicated). Data are depicted as mean +/- SD. *p<0.05, **p<0.01, ***p<0.001.

4.3 Determining whether mesenchymal stem cells promote corneal allograft survival in a hepatocyte growth factor-dependent manner

4.3.1 Research question

Is the capacity of MSCs to promote corneal graft survival dependent on their expression of HGF?

4.3.2 Experimental design

In order to determine whether suppression of HGF expression by MSCs limited their capacity to promote graft survival, we silenced HGF expression with small interfering RNA (siRNA). Details of siRNA transfection are provided in 2.9. Please note that the siRNA transfection was performed primarily by my colleague Sharad Mittal PhD at Schepens Eye Research Institute.[304] Subsequently, corneal transplantation was performed as detailed in 2.3. In addition to allogeneic grafts (C57BL/6 donors and BALB/c recipients), syngeneic grafts (BALB/c donors and BALB/c hosts) were performed to serve as controls. The allogeneic recipients were either (i) treated with

control siRNA (mock) MSCs at 3 hours following transplantation, (ii) treated with siHGF MSCs at 3 hours following transplantation, or (iii) left untreated. The number of MSCs administered was $0.5x10^6$ cells, suspended in 100uL sterile saline and delivered via tail vein injection, as described previously.[190,199,331] Graft opacity was assessed on a weekly basis by slit-lamp microscopy (Figure 4. 5), and transplant survival was determined according to the methods described in 2.3.

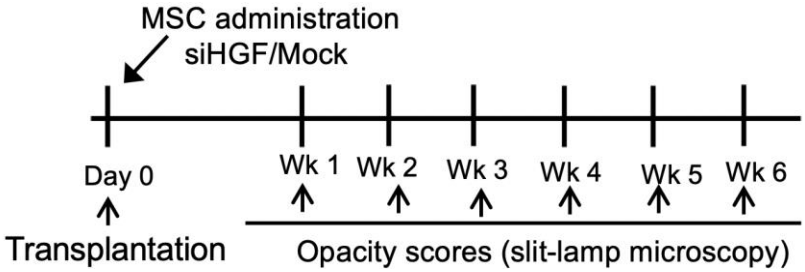

Figure 4. 5 Schematic of experimental design

4.3.3 Results

siRNA transfection resulted in an ~80% decrease in HGF expression by MSCs (Figure 4. 6). Corneal transplantation experiments demonstrated a two-fold increase in graft survival in mice that were administered mock MSCs (78%) relative to untreated controls. Notably, administration of siHGF MSCs exhibited a substantially reduced capacity to promote corneal graft survival (48%) relative to mock MSCs. These results clearly implicate MSC-derived HGF in promoting corneal graft survival.

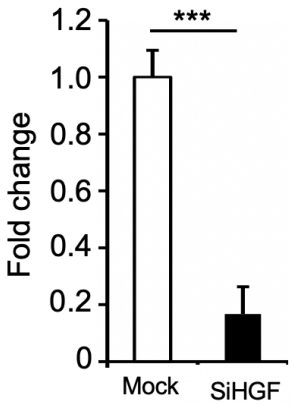

Figure 4. 6 Treatment of MSCs with HGF-specific siRNA reduces expression of HGF mRNA
Real-time analysis showing decreased expression of HGF mRNA after 72 hours of treatment of MSCs with HGF-specific siRNA (SiHGF), relative to control siRNA (Mock). Data are depicted as mean +/- SD. ***p<0.001.

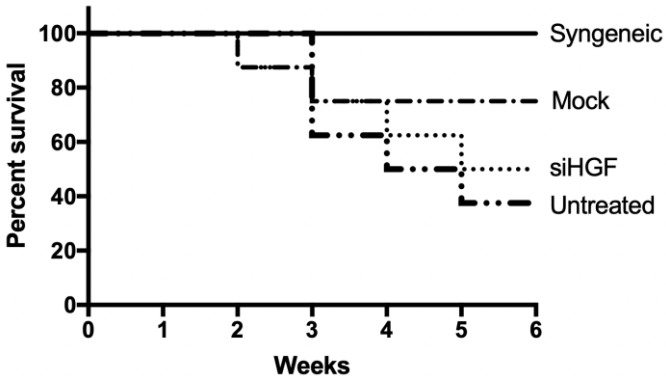

Figure 4. 7 MSCs promote corneal graft survival in an HGF-dependent manner

Kaplan-Meier analysis comparing graft survival in control siRNA (mock) MSC-injected recipients, siHGF MSC-injected recipients, untreated allogeneic recipients and untreated syngeneic recipients. n=8-10/group.

4.4 The effect of hepatocyte growth factor on induction of alloimmunity

4.4.1 Research question

Does treatment with HGF alone (independently of MSCs) suppress antigen-presenting cell activation and limit the generation of Th1 cells in allograft recipients?

4.4.2 Experimental design

In order to establish whether HGF alone can suppress the allogeneic immune response following corneal transplantation, we evaluated the *in vivo* effect of topical application of HGF on maturation of APCs, as well as on the generation of graft-attacking Th1 cells and tolerance-inducing Tregs. Corneal transplantation was performed, as described in 2.3. For two weeks postoperatively, either 5 μL of 0.1% murine recombinant HGF protein in PBS or protein control was applied topically to host eyes (Figure 4. 8).

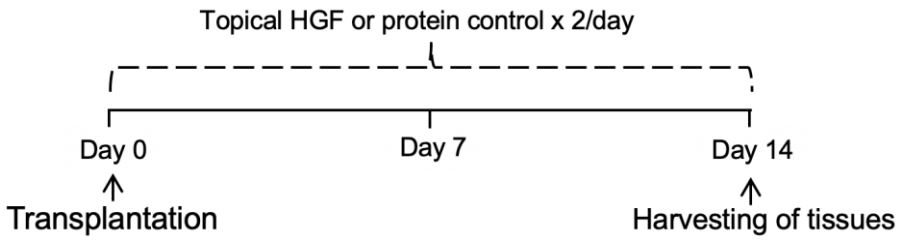

Figure 4. 8 Schematic of experimental design

At day 14 post-transplantation, draining lymph nodes were harvested and single cell suspensions were prepared. Flow cytometry was performed as described in 2.6. Fluorescently-conjugated antibodies against CD11c, MHCII, CD4, IFNγ and Foxp3 as well as their matched isotype controls were used.

4.4.3 Results

Corneal transplantation resulted in significantly increased frequencies of $CD11c^+MHCII^+$ cells in the draining lymph nodes of control-treated transplant hosts relative to naïve mice (p=0.015; Figure 4. 9). Notably, topical treatment with HGF substantially decreased frequencies of $CD11c^+MHCII^+$ cells compared to the control-treated recipients (p=0.006). There was a six-fold increase in frequencies of $CD4^+IFN\gamma^+$ Th1 cells in the lymph nodes of control-treated recipients compared to naïve mice (p=0.002; Figure 4. 10), yet this increase was abrogated by treatment with HGF. Indeed, treatment with HGF resulted in a 41% reduction in frequencies of $CD4^+IFN\gamma^+$ Th1 compared to control-treated recipients (p=0.033; Figure 4. 10). Given that $CD4^+Foxp3^+$ Tregs have been shown to promote graft tolerance, the effect of HGF treatment on frequencies of $CD4^+Foxp3^+$ Tregs was also assessed, and a moderate increase in Treg frequencies in the draining lymph nodes was observed relative to control-treated recipients (Figure 4. 11). Taken together, these results indicate that topical administration of HGF alone (independently of MSCs) suppresses the

alloimmune response, with inhibition of APC maturation and decreased Th1 generation.

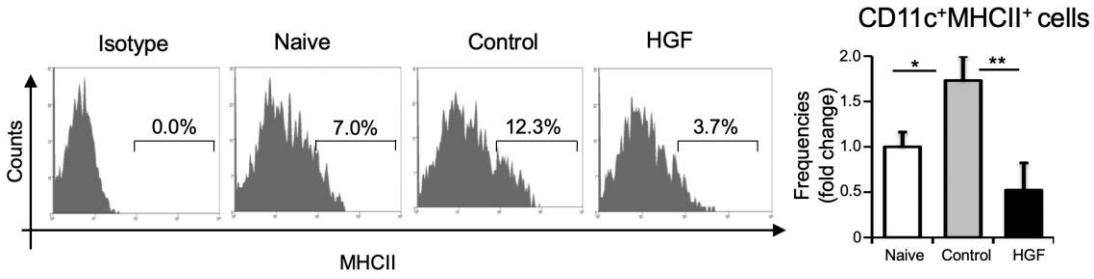

Figure 4. 9 Frequencies of CD11c$^+$MHCII$^+$ cells in the draining lymph nodes

Representative flow cytometry histograms (left) and summary bar chart (right) depicting relative frequencies of CD11c$^+$MHCII$^+$ cells in the draining lymph nodes of naïve, control and HGF-treated groups at Day 14 post-transplantation. n=4-6/group. Data are depicted as mean +/- SD. *p<0.05, **p<0.01.

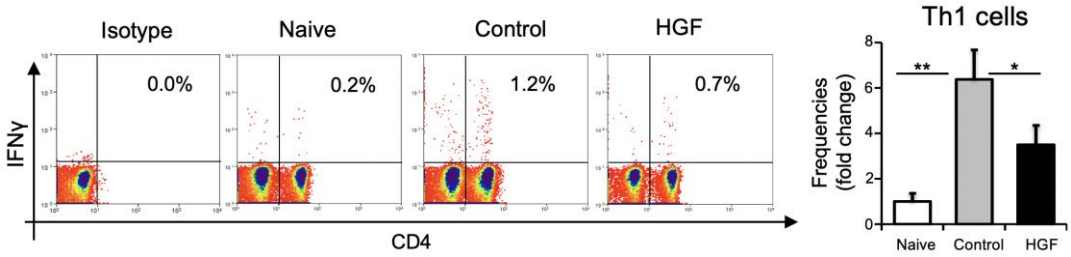

Figure 4. 10 Frequencies of CD4$^+$IFNγ$^+$ Th1 cells in the draining lymph nodes

Representative flow cytometry histograms (left) and summary bar chart (right) depicting relative frequencies of CD4$^+$IFNγ$^+$ Th1 cells in the draining lymph nodes of naïve, control and HGF-treated groups at Day 14 post-transplantation. n=4-6/group. Data are depicted as mean +/- SD. *p<0.05, **p<0.01.

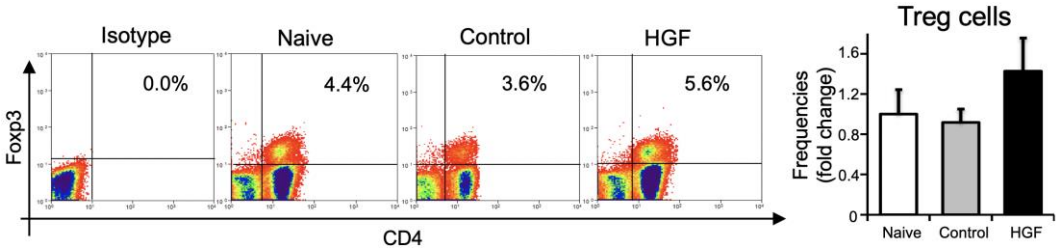

Figure 4. 11 Frequencies of CD4$^+$Foxp3$^+$ Th1 cells in the draining lymph nodes

Representative flow cytometry histograms (left) and summary bar chart (right) depicting relative frequencies of CD4$^+$Foxp3$^+$ Tregs in the draining lymph nodes of naïve, control and HGF-treated groups at Day 14 post-transplantation. n=4-6/group. Data are depicted as mean +/- SD.

4.5 The effect of hepatocyte growth factor on immune cell infiltration into corneal grafts

4.5.1 Research question

Does treatment with HGF alone (independently of MSCs) limit immune cell infiltration into corneal grafts?

4.5.2 Experimental design

Experimental design as detailed in 4.4.2. Corneas were harvested, digested, single cell suspensions were prepared, and flow cytometry was performed as described in 2.6. Fluorescently-conjugated antibodies against CD45, CD3 and CD11b as well as their matched isotype controls were used.

4.5.3 Results

Corneal transplantation resulted in an eight-fold increase in corneal infiltration of $CD45^+$ inflammatory cells in the control-treated group relative to naïve mice ($p=0.005$; Figure 4. 12). However, topical treatment of graft recipients with HGF reduced the frequencies of corneal $CD45^+$ cells by 52% ($p=0.026$). Compared to control-treated hosts, HGF-treated recipients demonstrated a 48% reduction in the corneal infiltration of $CD3^+$ T cells ($p=0.016$) and a 73% reduction in the corneal infiltration of $CD11b^+$ cells ($p=0.018$). In aggregate, these data demonstrate that topical application of HGF reduces graft infiltration of inflammatory cells following allogeneic corneal transplantation.

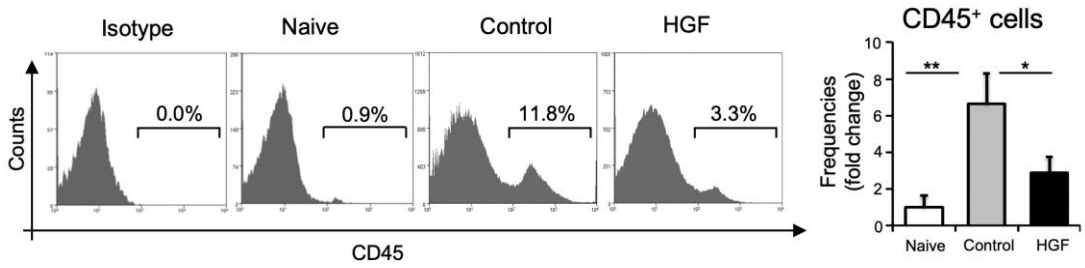

Figure 4. 12 Frequencies of CD45⁺ cells in corneal grafts

Representative flow cytometry histograms (left) and summary bar chart (right) depicting relative frequencies of CD45⁺ inflammatory cells in corneal tissue harvested from naïve, control and HGF-treated groups at Day 14 post-transplantation. n=4-6/group. Data are depicted as mean +/- SD. *p<0.05, **p<0.01.

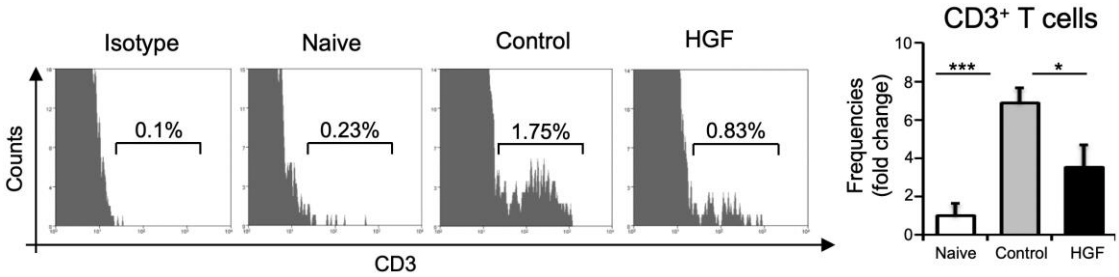

Figure 4. 13 Frequencies of CD3⁺ T cells in corneal grafts

Representative flow cytometry histograms (left) and summary bar chart (right) depicting relative frequencies of CD3⁺ inflammatory cells in corneal tissue harvested from naïve, control and HGF-treated groups at Day 14 post-transplantation. n=4-6/group. Data are depicted as mean +/- SD. *p<0.05, ***p<0.001.

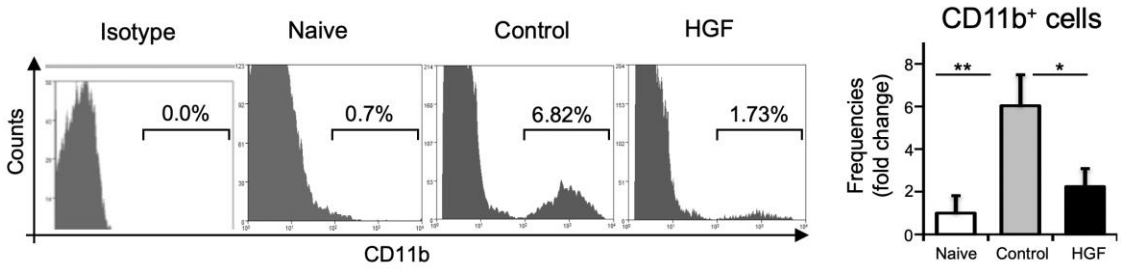

Figure 4. 14 Frequencies of CD11b⁺ cells in corneal grafts

Representative flow cytometry histograms (left) and summary bar chart (right) depicting relative frequencies of CD11b⁺ macrophages in corneal tissue harvested from naïve, control and HGF-treated groups at Day 14 post-transplantation. n=4-6/group. Data are depicted as mean +/- SD. *p<0.05, **p<0.01.

4.6 The effect of hepatocyte growth factor on corneal transplant survival

4.6.1 Research question

Does treatment with HGF alone (independently of MSCs) promote survival of corneal allografts?

4.6.2 Experimental design

In order to determine whether HGF alone can promote corneal allograft survival, we evaluated the *in vivo* effect of topical application of HGF on transplant survival. Corneal transplantation was performed, as described in 2.3. For two weeks postoperatively, either 5 µL of 0.1% murine recombinant HGF protein in PBS or protein control was applied topically to host eyes. Additional controls included untreated graft recipients and syngeneic graft recipients. Following this, graft opacity was assessed on a weekly basis by slit-lamp microscopy (Figure 4. 15), and transplant survival was determined according to the methods described in 2.3.

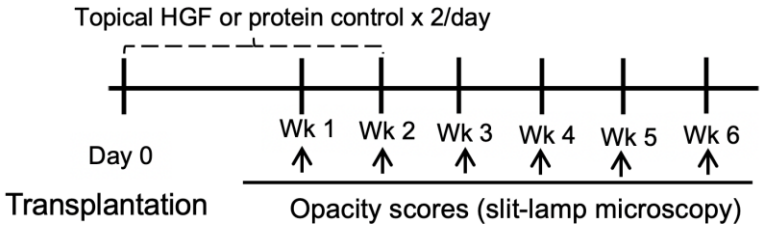

Figure 4. 15 Schematic of experimental design

4.6.3 Results

Untreated allograft recipients and allograft recipients treated with protein control exhibited ~40% transplant survival at week 6 post-operatively (Figure 4. 16). In

contrast, allograft recipients treated with topical HGF demonstrated 67% survival at week 6. No grafts were rejected in the syngeneic control hosts. These data indicate that, in addition to limiting the alloimmune response and corneal infiltration of inflammatory cells, topical HGF is effective in increasing corneal allograft survival.

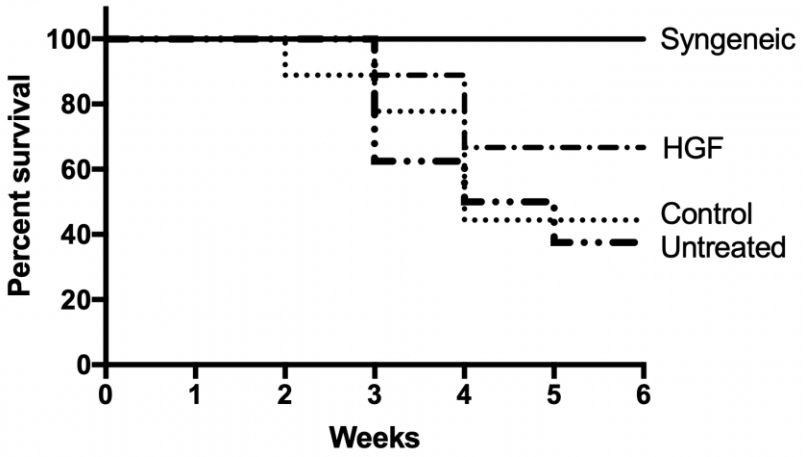

Figure 4. 16 Topical HGF promotes corneal allograft survival

Kaplan-Meier analysis comparing graft survival in topical HGF-treated allogeneic recipients, control-treated allogeneic recipients, untreated allogeneic recipients and untreated syngeneic recipients. n=8-10/group.

4.7 Discussion

The experiments described in this chapter add to our knowledge of the immunomodulatory mechanisms of MSCs in corneal transplantation. Specifically, the data show that: (i) an inflammatory milieu increases the expression of HGF by MSCs; (ii) MSC-derived HGF limits APC maturation and promotes corneal allograft survival; (iii) topical HGF alone (in the absence of MSCs) suppresses APC maturation, the generation of Th1 cells and the graft infiltration of inflammatory cells; and (iv) topical HGF alone promotes corneal transplant survival.

Published reports indicate that the immunoregulatory function of MSCs can be altered, and that the secretion of immunosuppressive mediators is upregulated by a local inflammatory microenvironment.[182,332] Our *in vitro* experiment demonstrates that MSC stimulation with IL1β results in increased secretion of the immunomodulatory factor HGF, and confirms findings from a previous study.[199]

During the alloimmune response, APCs are activated with increased expression of MHCII and co-stimulatory molecules such as CD80.[79,333] It has previously been reported that MSCs have the capacity to limit APC maturation.[334,335] In the setting of corneal allografts, it has been reported that systemic administration of MSCs at the time of transplantation reduces APC activation and promotes allograft survival.[190,192] By utilizing a soluble HGF receptor in our co-culture experiments, we have demonstrated that the inhibition of APC activation by MSCs is principally mediated by secretion of HGF.

Consistent with previous studies,[190,192] our data indicate that systemic administration of MSCs promotes corneal allograft survival. Indeed, we observed 75% graft survival at 6 weeks in hosts that were treated with MSCs transfected with control siRNA, in contrast with 38% survival in the untreated group. Yet the therapeutic benefit of MSCs was

reduced when MSCs transfected with *Hgf* siRNA were intravenously injected, with only 50% graft survival at 6 weeks. These *in vivo* data provide support for secretion of HGF being a key mechanism by which MSCs regulate the alloimmune response.

The immunomodulatory capacity of HGF has been demonstrated in various autoimmune diseases, including inflammatory bowel disease,[336] experimental autoimmune myocarditis,[337] allergic airway disease[338] and collagen-induced arthritis.[339] Furthermore, HGF has been shown to regulate the immune reponse in a murine model of heart transplantation, with reduced cellular infiltration of allografts, decreased myocardial necrosis and reduced IFNγ expression.[340] In a model of chronic graft-versus-host disease (GVHD), HGF gene transfer has been demonstrated to limit MHCII expression by recipient B cells and suppress DC immunogenicity, causing the amelioration of murine cholangitis, sialoadenitis and lupus.[341] In a model of acute GVHD, repeated transfection of human HGF cDNA into skeletal muscle has been shown to inhibit apoptosis of intestinal epithelial cells and reduce donor T cell infiltration into the liver, thus ameliorating the enteropathy and liver injury resulting from acute GVHD.[342] Thus there is mounting evidence for the immunoregulatory properties of HGF in a range of disease models.

There is considerable interest in the development of MSC-based therapeutics, both for ocular pathologies[171,343] and beyond.[344,345] Yet the delivery of MSC-based therapeutics is limited by factors common to all cell-based treatments: the technical challenge of consistently manufacturing clinical grade cells, and the financial and temporal challenges associated with a rigorous regulatory environment. As discussed, MSCs are known to mediate their immunomodulatory activity through a range of mechanisms, including the secretion of soluble factors.[346] The work presented in this chapter has shed light on the immunoregulatory function of one such factor, HGF, in the setting of corneal transplantation. The importance of HGF to MSC

immunoregulation is evidenced by the observation that promotion of corneal allograft survival by MSCs is reliant, in part, on the expression of HGF by MSCs. Moreover, our data indicate that blocking HGF *in vitro* severely compromises the suppression of IL1β-induced APC activation by MSCs.

Having established that HGF is a critical factor mediating the immunoregulatory activity of MSCs in corneal allotransplantation, the therapeutic efficacy of HGF alone was evaluated. These experiments are of high translational significance, since HGF mimetics do not face the stringent regulatory challenges associated with the development of cell-based therapies. The data presented in this chapter demonstrate that topical HGF limits APC activation and Th1 generation in the draining lymph nodes, reduces graft infiltration of inflammatory cells and promotes corneal transplant survival. Taken together, these results suggest that secretion of HGF represents a key mechanism by which MSCs exert their immunomodulatory activity, and suggest HGF as a potential therapeutic strategy to decrease the immune rejection of corneal allografts. Having demonstrated a potential role for HGF in modulating the antigen recognition phase of the adaptive immune response, I next set out to study a different phase of adaptive immunity – the generation of memory T lymphocytes.

CHAPTER 5. Dry eye disease: the role of memory T helper 17 cells in age-related dry eye disease

5.1. Introduction

5.1.1. Immunosenescence

The immune system changes in numerous ways during ageing, and these alterations are referred to in aggregate as 'immunosenescence'.[347] Although in some instances age-related changes to the immune response result in impaired function, this is not necessarily the case. Indeed, some facets of the immune response may demonstrate superior efficiency in ageing.[348] Yet taken together, these age-related alterations result a condition of chronic, sterile, low-grade inflammation that has been termed 'inflamm-ageing'.[349] This condition of inflamm-ageing predisposes older individuals toward overt inflammatory disease.[350]

The most apparent changes to the immune system during ageing affect adaptive immune responses (Figure 5. 1). Thymic involution occurs during ageing, and is known to accelerate following puberty.[351] As a result, older individuals have fewer naïve T cells, since there are lower frequencies of newly generated T cells egressing the thymus and entering the periphery.[352] Importantly, total numbers of T cells in the periphery do not decrease with age, due to compensatory age-related increases in effector and memory T cells relative to naïve T cells.[352] T cell mediated immunity is further impaired by narrowing of the T-cell repertoire,[353] reduced costimulatory signals[352] and cellular senescence.[354] Furthermore, there is evidence that B cells have impaired function with age, and that the repertoire of both $CD8^+$ T cells and Tregs are altered.[352]

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Figure 5. 1 Age-related changes to adaptive immunity

Ageing results in haematopoeitic stem cells (HSCs) skewing toward the myeloid rather than lymphoid lineage. Thymic involution occurs. Numbers of lymphocytes in the periphery are not reduced, however, due to an increase in memory T cells. T cells exhibit altered T cell receptor repertoires. From McKay and Jameson 2012

Ageing is also known to affect the innate immune system (Figure 5. 2). The ratio of myeloid DCs to plasmacytoid DCs is altered with advanced age,[355] and DCs derived from elderly individuals have been shown to have decreased phagocytic and migratory capabilities.[356] Ageing results in the decreased expression of MHCII, CD68 and CD14 by macrophages, which is associated with impaired function and responsiveness to lipopolysaccharide and IFNγ.[352] The function of neutrophils is also impaired with age, specifically their capacity for chemotaxis, phagocytosis and microbial killing.[357] Natural killer cells in ageing hosts have been shown to have reduced cytotoxic potential[358] with decreased production of proinflammatory cytokines and chemokines.[359]

Figure 5. 2 Age-related changes to innate immunity

Ageing alters the function of innate cells including macrophages, dendritic cells, neutrophils and natural killer cells. From McKay and Jameson 2012.

5.1.4. Age-related changes to the ocular surface

As we discuss the impact of immunosenescence, it is important to keep in mind the host of other changes that occur during ageing at the ocular surface.[360] Indeed, there is a reduction in goblet cell density[361] and tear production,[362] with lymphocytic infiltration and atrophy of the lacrimal gland.[363,364] Corneal nerve density and corneal sensitivity decrease with age.[365] Sex hormones are also reduced with age, which is known to impact the function of ocular surface glands.[366] Nevertheless, analysis of age-related alterations to human tear composition supports the notion of inflamm-ageing of the ocular surface, with older individuals exhibiting higher levels of proinflammatory and tissue remodeling factors in their tears compared to young individuals.[367] Furthermore, there is strong evidence implicating changes in the adaptive immune response as driving age-related DED from experimental murine models.[260,368]

5.1.5. Research questions

As a key characteristic of adaptive immunity, immunologic memory allows antigen-experienced lymphocytes to respond promptly and vigorously to repeat exposures to antigens.[369] Yet immunologic memory also contributes toward chronic inflammation in autoimmunity by the cognition of self-antigens.[255,370–373] As described, previous work from the Dana laboratory using a desiccating stress model of experimental DED has demonstrated that memory Th17 cells are crucial mediators of disease chronicity, and cause the increased disease severity that is observed when mice undergo secondary challenge with desiccating stress.[253] However, the role of memory Th17 cells in age-related DED is not known, and represents an important step in understanding how ageing predisposes toward ocular surface pathology. Indeed, key questions remain unanswered:

- Does increased age result in more severe corneal epitheliopathy following exposure to a dry environment?
- How does the Th17 immune response between young and aged mice differ when exposed to desiccating stress? Relatedly, how does the memory Th17 compartment compare?
- Are there potential therapeutic strategies to selectively target age-related aberrations of the immune response in DED?

To address these gaps in our current knowledge, I performed a series of experiments.

5.2. The effect of desiccating stress on corneal epithelial integrity in aged mice

5.2.1. Research question

What is the effect on corneal epithelial integrity of exposing young vs. aged mice to a dry environment?

5.2.2. Experimental design

In order to induce DED mice were housed in the controlled environment chamber, as described in 2.5.1. Corneal epitheliopathy was assessed by fluorescein staining, also described in 2.5.2. Young mice were aged 6-8 weeks on initial exposure to desiccating stress, and aged mice were aged 12-14 months, as described in 2.5.3.

For the primary challenge, mice were housed in the controlled environment chamber for 2 weeks. This duration was selected based on previous publications from the Dana laboratory for the induction of DED.[250,263,297] Following primary challenge, mice were housed in a standard vivarium for 3 weeks. Preliminary as well as previously published data indicated that 3 weeks was a sufficient duration for CFS score to fall to a steady 'chronic' CFS score.[253] Subsequently, mice underwent secondary challenge, and were returned to the controlled environment chamber for an additional 2 weeks before sacrifice.

5.2.3. Results

No difference was observed between young and aged mice in the change in CFS score (7 days: 4.3 [young] vs. 4.5 [aged], 14 days: 6.3 vs. 6.7 respectively). Yet on secondary challenge a significantly greater increase in corneal epitheliopathy was observed in aged mice relative to young mice (7 days: 5.5 [young] vs. 9.8 [aged] [$p<0.001$], 14 days: 8.1 vs. 11.1 respectively [$p=0.002$]). These data indicate that aged

mice exhibit a greater increase in disease severity compared to young on secondary, but not primary, exposure to desiccating stress.

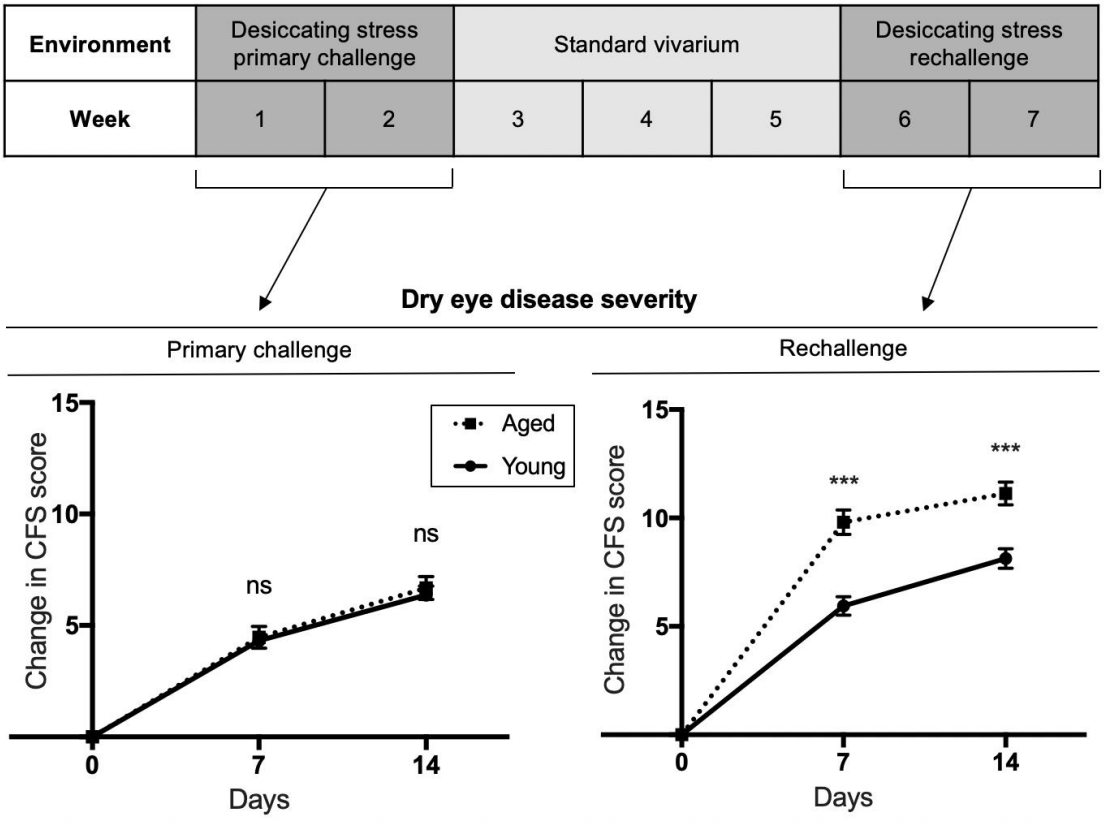

Figure 5. 3 Aged mice develop more severe corneal epitheliopathy relative to young on secondary, but not primary, exposure to desiccating stress

Schematic showing experimental design (upper). Corneal fluorescein staining (CFS) was used to evaluate corneal epitheliopathy during primary and secondary challenge (lower). No difference in change in CFS score was observed between young and aged mice during primary challenge, but on rechallenge significantly larger increases in CFS score were observed in aged mice relative to young at both day 7 and day 14. n=8/group. Data are depicted as mean +/- SEM. ***p<0.001.

5.3. The effect of desiccating stress rechallenge on Th17 immunity in aged mice

5.3.1. Research question

What is effect of desiccating stress rechallenge on the Th17 immune response in young vs. aged mice?

5.3.2. Experimental design

Mice were sacrificed following 14 days of desiccating stress rechallenge. Draining lymph nodes and conjunctivae were harvested, and single cell suspensions were prepared. Cells were stained with fluorescently-conjugated antibodies against CD4 and IL17A, as well as their matched isotype controls. Flow cytometry was performed as described in Chapter 2.

5.3.3. Results

In light of our data indicating severe corneal epitheliopathy in aged relative to young mice after rechallenge, we investigated frequencies of Th17 cells in the respective groups. As discussed earlier in this chapter, Th17 cells have been identified as the primary pathogenic effector cells in DED.[234,235,245]

Following rechallenge aged mice demonstrated substantially higher Th17 frequencies in the draining lymph nodes relative to aged naïve mice (4.2 ± 0.3% compared to 1.2 ± 0.3% respectively; p=0.004) (Figure 5. 4). Following rechallenge young mice had a tendency for higher Th17 frequencies in the draining lymph nodes compared to naïve, although this did not achieve statistical significance (1.4 ± 0.5 vs. 0.7 ± 0.3% respectively; p=0.248). Thus, aged mice demonstrated a 4.1 fold greater increase in Th17 frequencies in the draining lymph nodes compared to young (2.9% vs. 0.7% respectively).

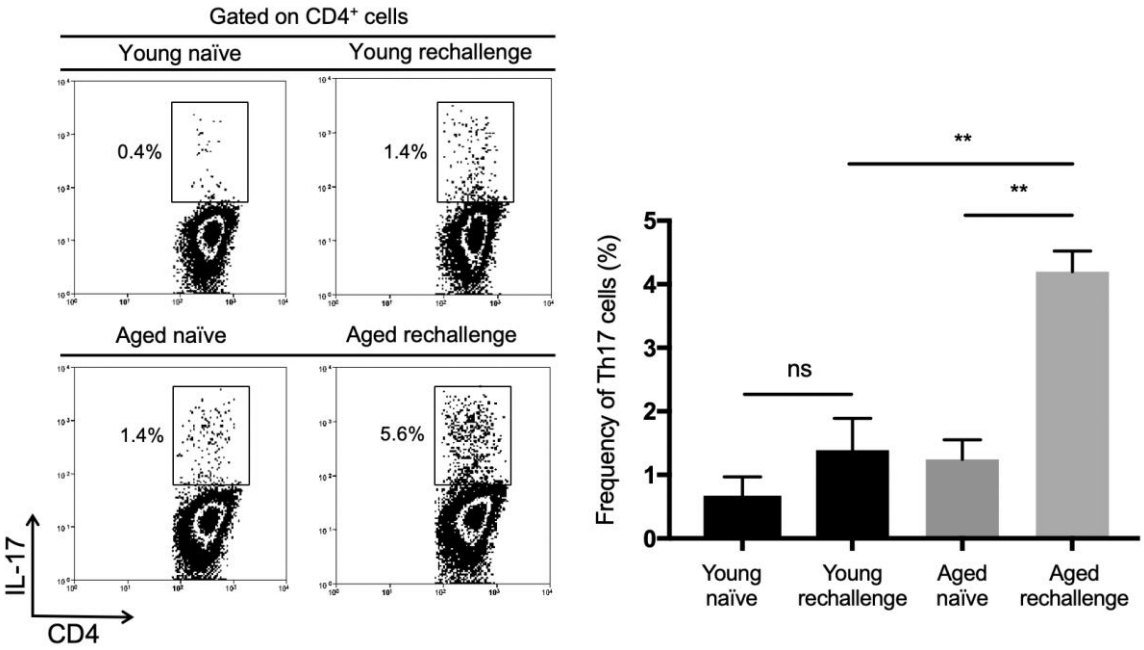

Figure 5. 4 Aged mice exhibit higher frequencies of effector Th17 cells in the draining lymph nodes compared to young following desiccating stress rechallenge

Representative flow cytometry dot plots (left) and bar chart (right) showing the frequencies of $CD4^+IL17A^+$ cells in the draining lymph nodes of young and aged mice, both naïve and following desiccating stress rechallenge. n=7-8/group. Data are depicted as mean +/- SEM. *p<0.05; **p<0.01.

Relative proportions of Th17 cells in the conjunctivae of young mice following rechallenge were amplified compared to naïve (6.6 ± 1.1% and 2.6 ± 0.6% respectively; p=0.011), similar to previous findings from the Dana laboratory[254] (Figure 5. 5). Notably, flow cytometry analysis of conjunctival single cells suspensions derived from aged mice showed a 2.1 fold greater increase in Th17 frequencies relative to young following rechallenge (8.3% in aged and 4.0% in young), with frequencies of 3.0 ± 0.7% in observed in naïve aged mice, and frequencies of 11.3 ± 1.4% detected following rechallenge (p<0.001).

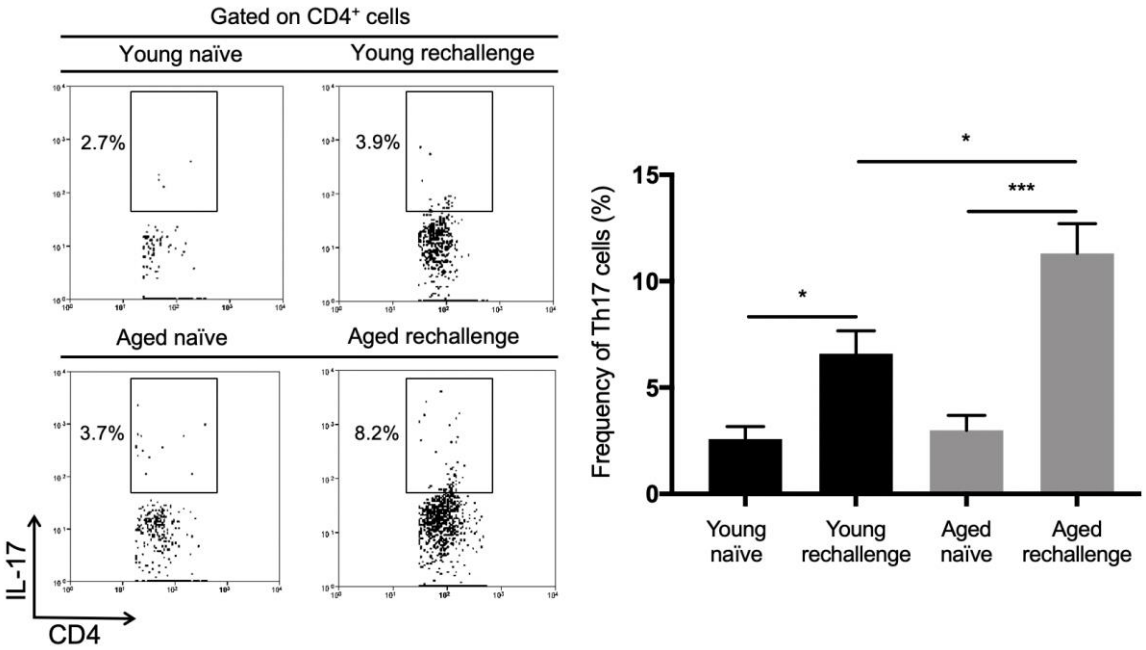

Figure 5. 5 Aged mice exhibit higher frequencies of effector Th17 cells in the conjunctivae compared to young following desiccating stress rechallenge

Representative flow cytometry dot plots (left) and bar chart (right) showing the frequencies of $CD4^+IL17A^+$ cells in the conjunctivae of young and aged mice, both naïve and following desiccating stress rechallenge. n=7-8/group. Data are depicted as mean +/- SEM. *p<0.05; **p<0.01.

5.4. The effect of systemic treatment with anti-IL-15 monoclonal antibody on the memory Th17 cell population in aged mice following primary challenge

5.4.1. Research question

What is effect of systemic administration of anti-IL-15 monoclonal antibody on the population of memory Th17 cells observed following desiccating stress primary challenge in young and aged mice?

5.4.2. Experimental design

Mice were exposed to desiccating stress for 2 weeks before being transferred to a standard vivarium. As shown in the schematic (Figure 5. 6), during the first of three weeks in the standard vivarium, mice were treated via intraperitoneal injection with either anti-IL-5 monoclonal antibody (25µg in 100µl of sterile saline) or 100µl sterile saline once daily for seven days. Following three weeks in the standard vivarium, mice were rechallenged for two weeks in the controlled environment chamber.

Draining lymph nodes and conjunctivae were harvested at two timepoints: after three weeks in the standard vivarium (i.e. prior to rechallenge) and following two weeks of rechallenge. Single cell suspensions were prepared. Cells were stained with fluorescently-conjugated antibodies against CD4, IL17A and CD44, as well as their matched isotype controls. Flow cytometry was performed as detailed in Chapter 2.

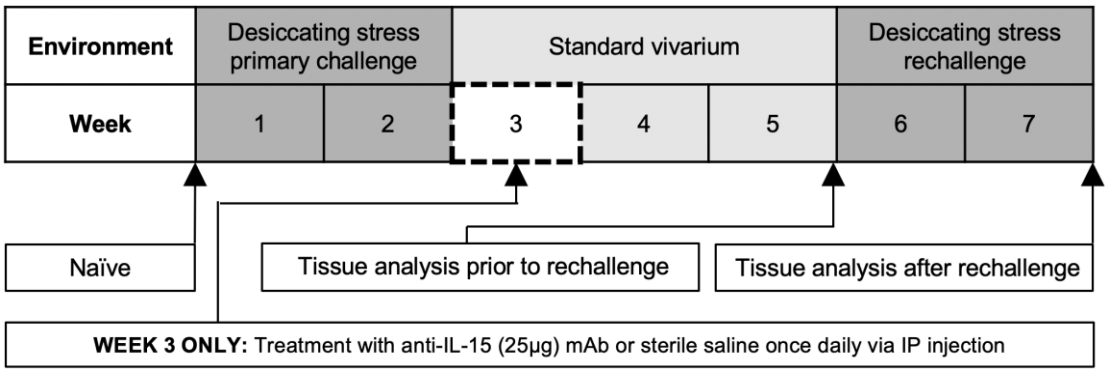

Figure 5. 6 Experiment schematic

5.4.3. Results

Published work has established that memory Th17 (mTh17; $CD4^+CD44^+IL17A^+$) are key mediators of inflammation in chronic DED.[253,254] In order to investigate the contribution of mTh17 cells to the severe exacerbations of DED observed in aged mice, I conducted an experiment in which anti-IL-15 mAb was used to deplete the mTh17 cell population in young and aged mice prior to rechallenge.

Analysis of tissues harvested before rechallenge demonstrated significantly higher mTh17 frequencies in aged saline-treated mice that had previously been exposed to desiccating stress compared to aged naïve mice (0.19 ± 0.07% vs. 0.10 ± 0.02% respectively, p=0.027) (Figure 5. 7). Still, this phenomenon was abrogated by systemic administration of anti-IL15 mAb, which was associated with a 4.6-fold decrease in mTh17 frequencies compared to aged PBS-treated animals (p<0.001). The young mice in our study exhibited similar trends, with higher frequencies in the saline-treated group compared to naïve and anti-IL-15-treated animals, but these differences did not reach statistical significance.

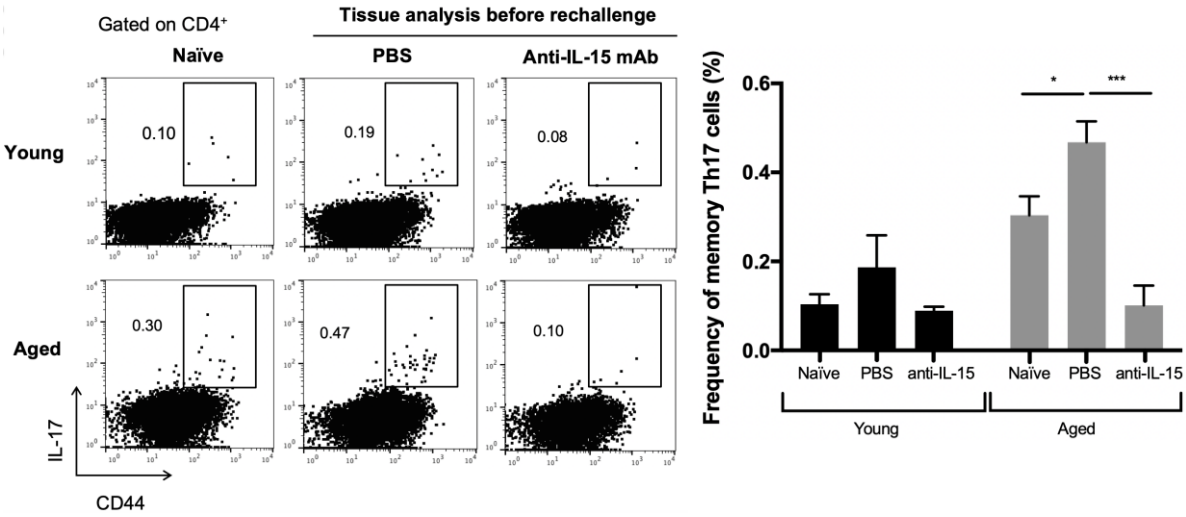

Figure 5. 7 Treatment with anti-IL-15 monoclonal antibody reduces the expanded memory Th17 cell population detected in aged mice after primary challenge

Representative flow cytometry dot plots (left) and bar chart (right) showing the frequencies of $CD4^+CD44^+IL17A^+$ cells in the lymph nodes of young and aged mice, both naïve and immediately prior to desiccating stress rechallenge, with and without anti-IL-15 mAb treatment (see experiment schematic for details). n=4-5/group. Data are depicted as mean +/- SEM. *p<0.05; ***p<0.001.

Interestingly, the frequencies of mTh17 cells detected in the lymph nodes of young and aged mice after systemic treatment with anti-IL-15 mAb were found to be similar (Figure 5. 8). These data indicate that treatment with anti-IL15 mAb effectively abrogates the difference in mTh17 cell frequencies between young and aged mice following primary exposure to desiccating stress.

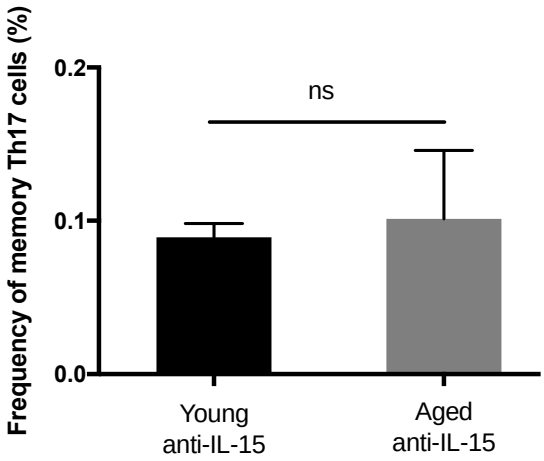

Figure 5. 8 Systemic treatment with anti-IL-15 monoclonal antibody after primary challenge results in comparable frequencies of mTh17 in the lymph nodes of young and aged mice

Bar chart depicting the frequencies of $CD4^+CD44^+IL17A^+$ cells in the lymph nodes of young and aged mice following treatment with anti-IL-15 mAb, immediately prior to desiccating stress rechallenge. n=4-5/group. Data are depicted as mean +/- SEM. *p<0.05.

5.5. The effect of systemic treatment with anti-IL-15 monoclonal antibody on severe dry eye exacerbations in aged mice

5.5.1. Research question

What is effect of systemic administration of anti-IL-15 mAb on DED severity and conjunctival effector Th17 frequencies in aged mice after desiccating stress rechallenge?

5.5.2. Experimental design

Experiment design was similar to that described in section 5.4 (as depicted in Figure 5. 6). After 2 weeks of desiccating stress, mice were transferred to a standard vivarium. Mice were administered either anti-IL-15 monoclonal antibody (25µg in 100µl of sterile saline) or 100µl sterile saline once daily for seven days via intraperitoneal injection. Following three weeks in the standard vivarium, mice were rechallenged for two weeks in the controlled environment chamber.

Following desiccating stress rechallenge, mice were sacrificed, and conjunctivae were harvested. Single cell suspensions were prepared. Cells were stained with fluorescently-conjugated antibodies against CD4 and IL17A, as well as their matched isotype controls. Flow cytometry was performed as described in Chapter 2.

5.5.3. Results

In view of the reduction in memory Th17 cell frequencies observed in aged mice following treatment with anti-IL-15 mAb, I next investigated the effect of systemic administration of anti-IL-15mAb antibody on DED severity. Consistent with my expectation that a depleted memory Th17 cell population would lead to reduced clinical disease following rechallenge, treatment with anti-IL-15 mAb resulted in significantly decreased DED severity in aged mice (Figure 5. 9). Despite a tendency

for lower CFS scores, treatment with anti-IL-15 did not significantly reduce DED severity in young mice.

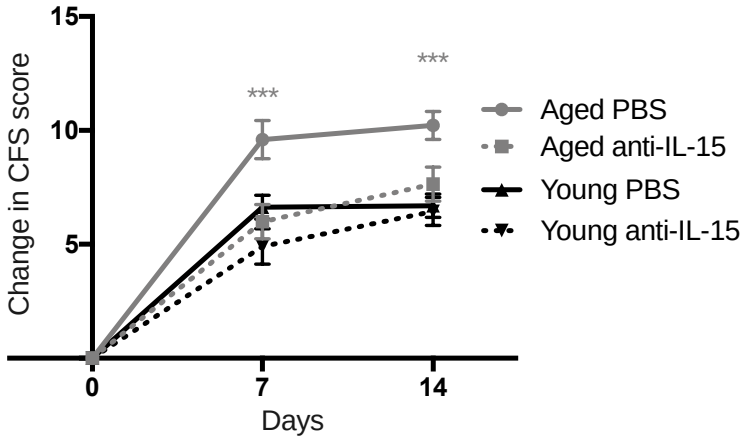

Figure 5. 9 Systemic administration of anti-IL-15 monoclonal antibody abrogates the severe dry eye exacerbations observed in aged mice

Treatment with anti-IL-15 mAb significantly reduced the increase in CFS score observed in aged mice following desiccating stress rechallenge at day 7 and day 14. In contrast, anti-IL-15-treated and saline-treated young mice exhibited similar increases in disease scores. n=7-8/group. Data are depicted as mean +/- SEM. ***p<0.001.

Mice were sacrificed at day 14 following rechallenge, and conjunctival frequencies of Th17 cells were analysed by flow cytometry. Lower frequencies of effector Th17 cells were detected in anti-IL-15 mAb-treated aged mice compared to saline-treated aged mice (5.1% and 10.1% respectively; p=0.016) (Figure 5. 10). Treatment of young mice with anti-IL-15 mAb did not result in a significant reduction in effector Th17 cell frequencies in the conjunctivae.

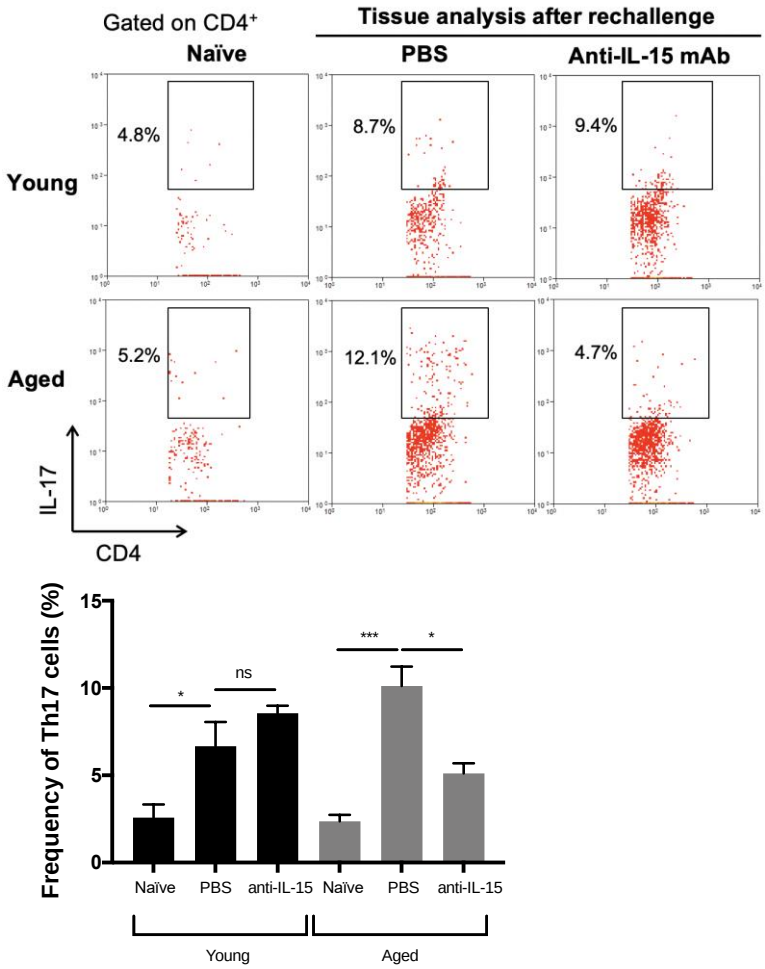

Figure 5. 10 Systemic administration of anti-IL-15 monoclonal antibody reduces effector Th17 frequencies observed the conjunctivae of aged mice

Representative flow cytometry dot plots (upper) and bar chart (lower) showing the frequencies of $CD4^+IL17A^+$ cells in the conjunctivae of young and aged mice, both naïve and following 2 weeks of desiccating stress rechallenge, with and without anti-IL-15 mAb treatment (see experiment schematic for details). n=7-8/group. Data are depicted as mean +/- SEM. *p<0.05; ***p<0.001.

5.6. Discussion

DED-associated morbidity is anticipated to increase in parallel with the enlarging older population.[206] There is a currently unmet need for therapeutics to relieve the burden of age-associated ocular surface disease. Similar to other chronic diseases for which increased age is a risk factor (such as rheumatoid arthritis[374] and psoriasis[375]), regulating the Th17 immune response has been proposed as a viable therapeutic strategy.[211] Notably, the disease course in these autoimmune conditions typically waxes and wanes, with mTh17 cells responding to putative self-antigens by rapid conversion to effector cells.[371,376] The data presented in this chapter shed light on differences in clinical DED severity and the Th17 immune response between young and aged mice. Consistent with previous work using an experimental autoimmune encephalitis model,[255] these data indicate that aged mice undergo more rapid and severe corneal epithelial disease following recurrent exposure to a dry environment compared to young mice. Moreover, these data show that treatment with anti-IL-15 monoclonal antibody reduces the memory Th17 population and limits the clinical disease observed in aged mice.

The desiccating stress model of DED employed in our study emulates several key facets of human disease, including corneal barrier disruption, elevated levels of Th17-related factors, secretion of pro-inflammatory cytokines by ocular surface epithelium and loss of goblet cells.[234,245,377–379] No difference in the increase in CFS score was observed between young and aged mice on primary exposure to desiccating stress. However, on repeat exposure to desiccating stress, aged mice exhibited more severe corneal epitheliopathy compared to young. Reports from other groups have described increased susceptibility to corneal epitheliopathy in aged animals.[260,368] Yet it is our understanding that the effect of rechallenge with desiccating stress (i.e. multiple exposures to a dry environment) has not previously been examined in murine ageing studies. In light of the clinical observation of increased DED severity in aged mice

compared to young following rechallenge, and based on the Dana laboratory's previous work showing the importance of memory Th17 cells to DED pathogenesis,[253,254] I speculated that an enlarged memory Th17 population in aged mice might be responsible for the more severe clinical disease detected in aged animals compared to young.

Work by Chen and colleagues has established the central role of memory Th17 cells in driving DED chronicity and has demonstrated that memory Th17 cells mediate the severe disease that results from rechallenge with desiccating stress.[253,254] As previously referenced, memory Th17 cells have also been reported to accelerate onset and increase disease severity in experimental autoimmune encephalitis, where memory Th17 cells have been observed to expand rapidly following rechallenge and migrate in high numbers to the central nervous system.[255] Yet the contribution of memory Th17 cells to age-related DED has not been established.

The data presented in this chapter demonstrate increased frequencies of effector Th17 cells in aged mice following rechallenge compared to young. Moreover, prior to rechallenge, an amplified pool of memory Th17 cells was observed in aged mice relative to young. Having made these observations, I sought to determine the effect of depleting the memory Th17 population in aged mice. IL-15 is a memory-maintaining cytokine, and previous studies have shown that targeting IL-15 signalling effectively depletes the memory Th17 cell pool.[254,380,381] Therefore, I conducted experiments in which young and aged mice were treated systemically with anti-IL-15 monoclonal antibody. Our data indicate that anti-IL-15 monoclonal antibody reduces the expanded memory Th17 pool in aged mice. Furthermore, anti-IL-15 monoclonal antibody was effective in reducing DED severity in aged mice that were rechallenged with desiccating stress, evidenced both by evaluation of clinical disease via corneal fluorescein staining and conjunctival frequencies of Th17 effector cells. Taken

together, these data indicate that an expanded pool of memory Th17 cells in aged mice predisposes these animals to severe DED exacerbations.

CHAPTER 6. General discussion of results

6.1. Introduction

Substantial progress has been made in recent decades in defining the anatomical, physiological and immunological features of the ocular surface and anterior segment that give rise to a particularly tolerant microenvironment.[50] Understanding these mechanisms is highly clinically significant, since excessive inflammation of the cornea results in opacification that is potentially blinding. Indeed, ten million people worldwide have bilateral corneal blindness, which is the third leading cause of blindness globally (after cataract and glaucoma).[1] Given the shortage of donor tissue worldwide (an estimated 53% of the world's population do not have access to corneal transplantation[2]), developing strategies to modulate the ocular immune response to both allogeneic donor tissue and corneal prosthetics is a clinical priority.

Dry eye disease (DED) is a highly prevalent disease of the ocular surface that reduces quality of life and is associated with a substantial socioeconomic burden.[203–205] The prevalence of DED increases with age; a large US population study has estimated that 0.2% of individuals aged 2-17 years are affected, rising to 11.7% for individuals older than 50 years.[206] As with autoimmune conditions such as inflammatory bowel disease,[247] multiple sclerosis,[249] rheumatoid arthritis,[248] and uveitis/scleritis,[246] Th17 cells have been shown to be the key immune effector cells in DED.[234,235,245] Moreover, published work from the Dana laboratory has implicated memory Th17 cells as driving DED chronicity, and predisposing toward severe disease exacerbations.[253] However, the role of memory Th17 cells in predisposing older individuals to DED has not been investigated.

Despite the progress that has been made in understanding immunomodulatory mechanisms in the eye, there are important unanswered questions that remain. The aims of this thesis have been concerned with investigating immunoregulation at two

specific phases of the adaptive immune response – antigen recognition and generation of memory (as highlighted in Figure 6. 1).

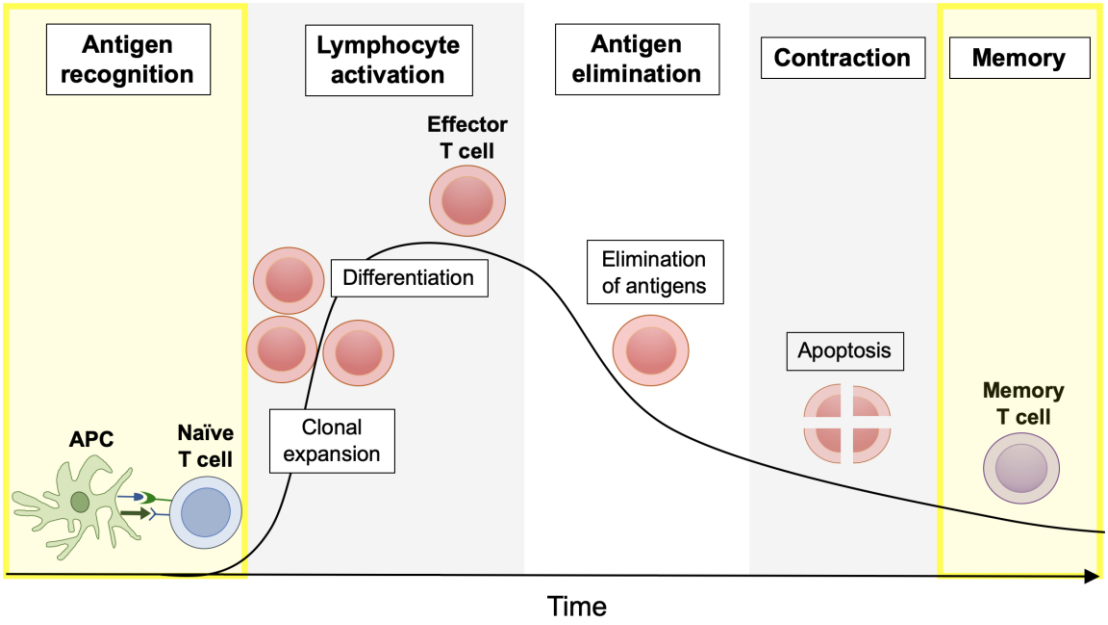

Figure 6. 1 Phases of the adaptive immune response

The adaptive immune response consists of distinct phases. The adaptive immune response is initiated when antigens are ingested by immature antigen-presenting cells, which subsequently undergo maturation. The mature antigen-presenting cells migrate to a regional lymph node, where they activate lymphocytes. Effector lymphocytes eliminate antigen. During contraction, antigen-stimulated lymphocytes undergo apoptosis, with the exception of subset of antigen-specific cells that form the memory pool. The phases of the immune response that this thesis will focus on (antigen recognition and the generation of memory) are highlighted.

The murine model of allogeneic corneal transplantation[164,165,168,261] was selected as an appropriate model to investigate immunoregulation at the antigen recognition phase of the adaptive immune response. Advantages of this model include clearly defined antigenic load (in contrast with model of DED) and the extensive experience of the Dana laboratory.[164,267,382–384] Furthermore, this model permits evaluation of graft opacity (and thus graft failure) without necessitating the sacrifice of the host, unlike other models of transplantation.

A desiccating stress murine model of DED was selected to investigate the role of memory Th17 cells in ageing. This model was chosen due to the fact that previous work has shown that a substantial population of memory Th17 cells is formed in

female C57BL/6 mice following exposure to desiccating stress, and that these cells are highly pathogenic.[253,254] The Dana laboratory also has many years of experience of working with this model.[250,262–264]

The rationale for using anti-IL-15 to deplete the memory Th17 pool was based on previous work from the Dana laboratory.[254] Chen and colleagues demonstrated that in the desiccating stress model of dry eye, treatment with either anti-IL7 or anti-IL15, but not anti-IL17 or control IgG, was successful in depleting mTh17 frequencies both locally at the ocular surface and at the draining lymph nodes.[254] In this work, the authors show that IL7 and IL15 signaling is required for the maintenance of immunopathogenic memory Th17 cells. Given that treatment with either anti-IL7 or anti-IL15 would be effective in reducing mTh17 frequencies, and in view of the pleiotropic immunoregulatory activities of IL7,[385] in my experiments I employed anti-IL15 to deplete the mTh17 pool.

During the course of the described experiments, I aimed to employ these two models to further characterize specific features of the ocular inflammatory response during the antigen recognition and memory phases of the adaptive immune response. Furthermore, I sought to investigate potential therapeutic strategies to decrease pathogenic corneal inflammation in these settings. Firstly, I aimed to characterize the effect of the purinergic receptor antagonist oxidized adenosine triphosphate on corneal allograft survival. Next, I sought to determine the role of MSC-derived hepatocyte growth factor in the promotion of corneal allograft survival. Finally, I conducted experiments to determine the contribution of memory Th17 cells to the severe dry eye disease observed in aged mice.

6.2. Investigating the antigen recognition phase of the adaptive immune response using a murine model of corneal transplantation: the effect of the purinergic receptor antagonist oxidized adenosine triphosphate on immune-mediated graft rejection

6.2.1. Summary and discussion of my findings

In the experiments described in Chapter 3, I investigated the immunomodulatory effects of oxidized ATP, an irreversible antagonist of ATP, in the setting of corneal allotransplantation. The data demonstrate that systemic administration of oATP to corneal allograft recipients promotes transplant survival. The data also indicate that treatment with oATP reduces corneal infiltration of inflammatory cells, suppresses APC maturation, decreases frequencies of Th1 cells in the draining lymph nodes and decreases graft infiltration by $CD4^+$ T cells. oATP was not observed to have a significant effect on either Treg frequency or function.

I became interested in the purinergic receptor antagonist oATP following the publication of an article in the journal Kidney International in August 2017, entitled 'The P2X7 receptor antagonist, oxidized adenosine triphosphate, ameliorates renal ischemia-reperfusion injury by expansion of regulatory T cells'.[318] In this article, the investigators demonstrated the therapeutic effects of administering oATP both prior to and after renal ischemia-reperfusion injury, with decreased blood urea nitrogen and serum creatinine observed in the treated groups relative to control. Renal infiltration of $CD4^+Foxp3^+$ Tregs was detected to be increased following treatment with oATP, and interestingly, depletion of Tregs in wild-type mice was observed to abrogate the beneficial effects of oATP. oATP is known to antagonize the activation of P2X7 receptors,[386] and Koo and colleagues employed P2X7R knockout mice in a series of experiments to demonstrate that oATP mediated the expansion of regulatory T cells and reduced renal ischemia-reperfusion injury by blocking the activation of P2X7R.[318]

I found the article by Koo and colleagues to be particularly intriguing in the context of recent work published by the Dana laboratory.[155,159,162–165] Multiple studies had clearly demonstrated the capacity of Tregs to promote allograft survival. For example, work by Tahvildari and colleagues had shown that low-dose systemic administration of IL-2 in the peri-transplantation period increased both the frequencies and immunosuppressive function of Tregs, and resulted in improved allograft survival.[165] Furthermore, local delivery of exogenous Tregs had been shown to promote transplant survival by Shao and colleagues.[164] In this work, GFP^+ Tregs were isolated from GFP^+ transgenic mice, injected subconjunctivally into corneal allograft recipients at the time of corneal transplantation. GFP^+ Tregs were detected in the ipsilateral cornea and DLNs of graft recipients at 6 hours after injection. In addition to promoting graft survival, conjunctival delivery of Tregs was observed to suppress frequencies of mature APCs and Th1 cells in the draining lymph nodes, decrease expression of IFNγ in the graft and increase graft expression of the Treg-associated cytokines IL-10 and TGFβ.[164]

The report from Koo and colleagues[318], combined with reports from the Dana laboratory on the capacity of Tregs to promote tolerance in the setting of corneal transplantation[155,159,162–165], led me to ask the question: 'Would oATP promote corneal transplant survival by expanding regulatory T cells?'. Fortunately, I was able to refer to the work performed by Paulo Fiorina's laboratory, who had published three reports on the role of P2X7R in transplantation using models of cardiac, lung and islet cell grafting.[315–317] In each of these papers, the group demonstrated that targeting P2X7R with oATP promoted allograft survival. Other studies had investigated the effect of oATP treatment in models of autoimmunity.[387–389] However, there was a lack of consensus between these reports on the effect of oATP on Tregs, with some reports describing increased Treg activity[315,318,387,388], and other studies reporting minimal

effect on Tregs.[316,317,389] Thus, in order to clarify the effect of oATP on corneal allograft survival and the Treg pool, I set about conducting preliminary studies.

During my preliminary investigations I detected very similar frequencies of Tregs in the draining lymph nodes and corneas of oATP-treated compared to saline-treated mice or naïve mice. Levels of Foxp3 expression by Tregs have been shown to be indicative of their functional status in corneal transplantation.[155] In my preliminary investigations, Foxp3 expression (MFI) was observed to be similar across the groups. Furthermore, no difference in suppressive capacity of oATP-treated versus untreated Tregs was observed in *in vitro* studies.

Nevertheless, I had conducted survival experiments which clearly demonstrated the capacity of systemic treatment with oATP to reduce corneal allograft opacification and prolong survival. Moreover, evaluation of corneal tissue clearly demonstrated reduced infiltration of $CD45^+$ inflammatory cells and $CD4^+$ T cells in the oATP-treated mice, compared to saline-treated animals. Thus, the focus of my investigation changed to how oATP was limiting the effector arm of the alloimmune response, rather than amplifying the regulatory arm. Additional data demonstrated substantial reductions in the frequencies of mature APCs at both the graft site and the draining lymph nodes of the oATP-treated recipients, with concomitant suppression of Th1 frequencies. These data clearly indicated that oATP was limiting the activation of APCs following corneal transplantation, yet the question remained as to whether oATP was also having a direct (i.e. APC-independent) on T cells. To resolve this question, I conducted mixed lymphocyte reactions in which oATP- or PBS-treated T cells were co-cultured with oATP-treated or PBS-treated APCs. These data indicated that, while oATP did have a direct effect on T cells, the effect on APCs was the dominant of these two phenomena. In further support of this observation, when APCs were treated with oATP, the levels of

dilution of CFSE-labeled cells were similar regardless of whether or not T cells were treated with oATP.

In summary, although my initial interest in oATP was on its capacity to modulate Tregs, the focus of these investigations ultimately became how oATP regulates APC activation and Th1 immunity. The Fiorina group did not evaluate oATP-mediated suppression of APC maturation in the setting of allotransplantation.[315–317] Accordingly, these data are the first to report that a fundamental mechanism of oATP-mediated modulation of the immune response in allogeneic transplantation is the limitation of APC activation.

6.2.2. Future work

There are several questions that have been raised by this work, which I am keen to pursue. First of all, there are questions directly related to the experiments I performed. These include studying the effect of oATP treatment on other immune cells. Although previous work has shown that activation of $CD11b^{+}MHCII^{+}$ cells and the subsequent infiltration of graft-attacking $CD4^{+}IFN\gamma^{+}$ Th1 cells play a crucial role in determining immune rejection,[134,137,138,322,329,390] it is likely that oATP also impacts the function of other inflammatory cells, that in turn contribute to immune rejection. A survey of the effect of oATP on other inflammatory cell types involved in corneal graft rejection would deepen our understanding of oATP-mediated immunomodulation.

Another question that I am keen to pursue concerns the effect of oATP on immune cell frequencies at different timepoints. Previous studies have demonstrated that 2 weeks is an optimum time point to evaluate the alloimmune response prior to the onset of graft rejection.[261,320,330] Thus, in my experiments I evaluated immune cell frequencies at this timepoint. However, analysing the kinetics of immune cells following oATP treatment would further elucidate oATP's immunomodulatory activities. Indeed,

previous work has shown that increased inflammation at the graft site (e.g. using a model of high-risk transplantation in which corneal inflammation and neovascularization is induced by placing stromal sutures two weeks prior to transplantation) results in increased APC maturation as early as three days following grafting.[390] Additional timepoints to evaluate the kinetics of immune cells might include day 1, day 3, day 7, day 28 and day 56 post-transplantation.

In addition, there are experiments that are indirectly related to the experiments I performed, but would further illuminate the mechanisms of action of oATP in corneal transplantation. For example, I am keen to study whether the immunosuppressive functions of oATP in corneal transplantation are mediated via P2X7R. P2X7Rs are unusual amongst purinergic receptors as they have relatively low affinity for ATP and are thus only activated in highly inflamed tissues.[307] Furthermore, P2X7R can form pores that permit the passage of molecules as large as 900 daltons, which includes ATP itself.[307] oATP is known to antagonize P2X7R, but there is evidence that it has anti-inflammatory effects that are independent of P2X7R.[391] In order to demonstrate conclusively that oATP was mediating its immunomodulatory activity via P2X7R, I would like to perform experiments employing P2X7R knockout mice, which are commercially available.

6.3. Investigating the antigen recognition phase of the adaptive immune response using a murine model of corneal transplantation: the effect of mesenchymal stem cell-derived hepatocyte growth factor on immune-mediated graft rejection

6.3.1. Summary and discussion of my findings

I have conducted experiments investigating the immunomodulatory mechanisms of MSCs in corneal transplantation. The data resulting from these experiments show that the expression of the pleiotropic growth factor HGF by MSCs is increased in an inflammatory microenvironment, and that HGF derived from MSCs inhibits APC maturation and increases corneal allograft survival. Furthermore, our data indicate that topical HGF alone (in the absence of MSCs) limits APC maturation, decreases Th1 generation, suppresses inflammatory cell infiltration of the cornea and promotes allograft survival.

The genesis for these investigations was a previous report from the Chauhan laboratory entitled 'Mesenchymal stem cells home to inflamed ocular surface and suppress allosensitization in corneal transplantation', published in Investigative Ophthalmology & Visual Science in 2014.[190] In this paper, Omoto and colleagues demonstrated that GFP-labelled MSCs injected intravenously at three hours post-transplantation were found in abundance in the grafted cornea, ipsilateral conjunctiva and lymph nodes at 3 days post-transplantation, but not in the contralateral (ungrafted) ocular surface or lymph nodes. Furthermore, the authors demonstrated that the lymph nodes of recipients treated with MSCs had lower frequencies of IFNγ-secreting Th1 cells relative to control mice. Finally, graft survival rates in the MSC-treated group was significantly higher compared to the control mice.[190] However, this report did not identify specific MSC-derived factors that modulated the immune response.

Work by other laboratories had demonstrated the capacity of MSCs to promote corneal allograft survival.[192–194] Notable amongst these studies was a report published by Darwin Prockop's group, since the investigators observed that rather than migrating to the cornea, the majority of intravenously-administered MSCs became trapped in the lungs, and exerted their immunomodulatory function via the secretion of tumor necrosis factor-α stimulated protein 6 (TSG-6).[192] However, the investigators were using human-derived rather than mouse-derived MSCs in their experiments, and thus it is possible that the inconsistency between reports from the Chauhan and Prockop laboratories was related to issues of engraftment and integration when cells were administered across the xeno-species barrier.

Previous screening experiments conducted by Mittal and colleagues have established that stimulation of *in vitro* expanded and functionally characterized bone marrow-derived MSCs with IL-1β (to emulate inflammatory conditions) for 24 hours results in a moderate increase in expression of *Tsg-6* (as determined by real-time PCR), but promotes a far greater increase in expression of *Hgf.*[199] Moreover, the >2-fold increase in *Hgf* expression observed with real-time PCR was corroborated by performing ELISA on culture supernatants, which demonstrated a >2-fold increase in HGF secretion by IL-1β-stimulated MSCs.[199] In experiments employing *Hgf*-specific siRNA to downregulate HGF expression on MSCs that were subsequently administered via intravenous injection into mice with corneal injuries, Mittal and colleagues demonstrated that MSC-derived HGF inhibited opacification following injury.[199] However, the capacity of MSC-derived HGF to modulate the alloimmune response and promote corneal graft survival had not previously been investigated. In order to address that gap in our current knowledge, we conducted a series of experiments.

Our data clearly show that MSCs inhibit APC maturation through the secretion of HGF, as demonstrated by co-culture experiments in which HGF was neutralized using

soluble HGFR and the expression of MHCII and CD80 was evaluated. Our results corroborate previous reports that MSCs promote corneal allograft survival,[192–194] but also show that this phenomenon is dependent on the expression of HGF. We observed that topical treatment with HGF alone (without MSCs) in the two-week post-transplantation period suppressed APC maturation and decreased frequencies of graft-attacking Th1 cells in allograft recipients. Moreover, treatment with HGF alone decreased immune cell infiltration of the corneal graft and promoted transplant survival.

6.3.2. Future work

Previous work by the Chauhan laboratory has demonstrated that MSCs migrate specifically to the graft site following corneal allotransplantation, where they promote graft survival.[190] Moreover, the Chauhan laboratory has established that the therapeutic effects of MSCs in the setting of corneal injury are dependent on their expression of HGF.[199] The experiments described in Chapter 4 address the question that is arises naturally from reading these two papers; that is, whether the immunoregulatory function of MSCs in corneal transplantation is also contingent on expression of HGF? The data presented demonstrate that MSCs modulate the immune response in corneal transplantation via secretion of HGF and indicate the therapeutic potential of HGF alone in reducing the immune rejection of corneal grafts.

Future work on the immunomodulatory properties of HGF in corneal transplantation might include a study to evaluate the effect of HGF administration in high-risk transplantation. Indeed, low-risk transplants conducted in non-vascularized and uninflamed host beds enjoy graft survival rates of >90%.[110] The clinical need for novel therapeutics to improve graft outcomes is in the high-risk setting, where inflammation and vascularization of the host bed result in rejection rates that exceed 50%.[109,111] Data indicating that HGF improved graft survival in the high-risk murine model of

corneal transplantation (in which stromal sutures are placed two weeks prior to transplantation to induce vascularization) would be valuable. This is particularly the case given that HGF has been reported to have promote angiogenesis,[392,393] which is a risk factor for immune rejection.[79] Furthermore, in our experiments topical HGF was administered to the ocular surface twice daily for two weeks post-transplantation. Additional data demonstrating the effects of administering HGF according to different dosing schedules and durations would assist future studies.

6.4. Investigating the memory phase of the adaptive immune response using a murine model of dry eye disease: the role of memory Th17 cells in age-related dry eye disease

6.4.1. Summary and discussion of my findings

In investigating the contribution of memory Th17 cells to age-related dry eye disease (DED), I used a desiccating stress murine model of DED to demonstrate that: (i) aged mice are prone to more severe disease exacerbations compared to young, (ii) aged mice have an expanded memory Th17 pool relative to young following primary exposure to desiccating stress, and (iii) systemic administration of anti-IL-15 monoclonal antibody depletes the memory Th17 cell population prior to rechallenge, abrogates the increased effector Th17 response observed in aged mice after rechallenge, and reduces DED severity in aged mice.

The premise for the experiments described in Chapter 5 was the clinical observation that the prevalence of DED increases substantially with age, from 0.2% for ages 2-17 years to 11.7% for ages more than 50 years.[206] The immune system is critically implicated in driving the self-perpetuating cycle of inflammation that characterizes DED pathogenesis.[211] Yet the immune system is known to change in numerous ways during ageing, collectively termed 'immunosenescence'.[347] These age-associated alterations to the immune response give rise to 'inflammageing' – a condition of sterile, low-grade inflammation that predisposes to overt inflammatory disease.[349,350] In view of the fact that the immune system plays a major role in DED pathology, and the numerous changes to the immune system that occur with age, I speculated that age-related variations in clinical DED may be secondary to disparities in the immune response.

The initial experiments I conducted involved exposing young and aged mice to desiccating stress and using CFS staining to score the resultant corneal epitheliopathy at various timepoints. Interestingly, I did not initially observe a difference in CFS score

between young and aged mice exposed to desiccating stress (i.e. during primary challenge). Rather than sacrifice the mice, I returned the mice to a standard vivarium and continued to monitor CFS scores. But again, the scores were similar. It was only when I rechallenged the mice with desiccating stress (i.e. secondary challenge) that a difference in clinical disease between young and aged became apparent – the aged mice developed severe corneal epitheliopathy faster than the young mice. The observation that aged mice developed severe disease after previous exposure to desiccating stress suggested that immune memory might be contributing to this phenomenon.

Th17 cells are recognized as the major effector immune cells in DED.[234,235,245] In addition, Th17 cells have been shown to have shown to promote autoimmune disease in uveitis/scleritis,[246] inflammatory bowel disease,[247] rheumatoid arthritis[248] and multiple sclerosis.[249] Yet Th17 cells also provide critical protection against commensals and opportunistic infections at mucosal surfaces, and have been shown to promote wound repair.[394] Certainly, there is evidence to suggest that Th17 cells have tissue-specific functions.[395] With this in mind, it is interesting to note that the eye and the brain are immune privileged sites that exhibit a relatively quiescent immune environment,[396,397] and that Mandy McGeachy's group, employing a model of experimental autoimmune encephalitis, has demonstrated that memory Th17 cells drive earlier onset and increased severity of disease following rechallenge.[255] Previous work has shown that memory Th17 cells are highly pathogenic in the eye (even more so than effector Th17 cells) and give rise to severe DED exacerbations with accelerated disease onset following rechallenge.[253,254] Based on these reports, I designed experiments to examine how the memory Th17 pool might influence DED severity in young and aged mice.

Examination of the memory Th17 pool *prior to rechallenge* revealed significantly higher frequencies in aged mice relative to naïve controls. Moreover, this correlated with substantially higher frequencies of effector Th17 cells in the lymph nodes and conjunctivae of aged mice *after rechallenge* compare to naïve controls. In order to investigate whether memory Th17 cells were driving the severe DED exacerbations observed in aged mice, I conducted experiments in which mice were treated systemically with anti-IL-15 monoclonal antibody to deplete memory Th17 cells. Previous studies had shown that targeting IL-15 signalling was an effective strategy to deplete the memory Th17 cell pool.[254,380,381] Systemic administration of anti-IL-15 monoclonal antibody was observed to reduce the expanded memory Th17 pool and reduce the severity of DED exacerbations in aged mice.

In sum, the work presented in Chapter 5 was instigated by the clinical observation of age being a risk factor for DED. Ageing modulates the immune system in numerous ways, and despite a general decline in immunocompetence (increased susceptibility to infection, inadequate responses to vaccination, impaired suppression of neoplasia), elderly individuals are paradoxically also predisposed to certain autoimmune diseases (e.g. rheumatoid arthritis[374] and psoriasis[375]). The data presented in Chapter 5 indicate that memory Th17 cells contribute to the increased corneal epithelial disease observed in aged animals following repeated exposure to desiccating stress.

6.4.2. Future work

Although the experiments presented in Chapter 5 strongly suggest that memory Th17 cells promote the increased disease severity observed in aged mice, they do not definitively prove this to be the case. Using anti-IL-15 monoclonal antibody is a non-specific approach for the depletion of memory Th17 cells, which has numerous effects on other types of immune cell. For example, IL-15 is known to play a role in the activation and proliferation of NK cells.[398] Additional experiments are required to

demonstrate causality between the amplified memory Th17 cell pool observed in aged mice and enhanced DED severity. For example, adoptive transfer experiments might be performed in which the memory Th17 compartment is isolated from young and aged mice following primary exposure to desiccating stress, and adoptively transferred into young, naïve mice. These recipient mice would then be rechallenged with desiccating stress, with CFS scores evaluated and tissues harvested for analysis of effector Th17 cells by flow cytometry. Based on the data presented in Chapter 5, I would predict that young mice that had received adoptive transfer of the memory Th17 pool derived from aged mice would have similar CFS scores and immune assay readouts to aged mice following rechallenge.

I would also be interested in investigating age-related alterations in Treg function in the setting of DED. In preliminary experiments conducted during my PhD studies, I have observed that frequencies of Tregs are increased in aged mice relative to young (Figure 6. 2). Other data indicate that after 2 weeks of desiccating stress, aged mice exhibit decreased expression of Foxp3 and decreased suppressive function.

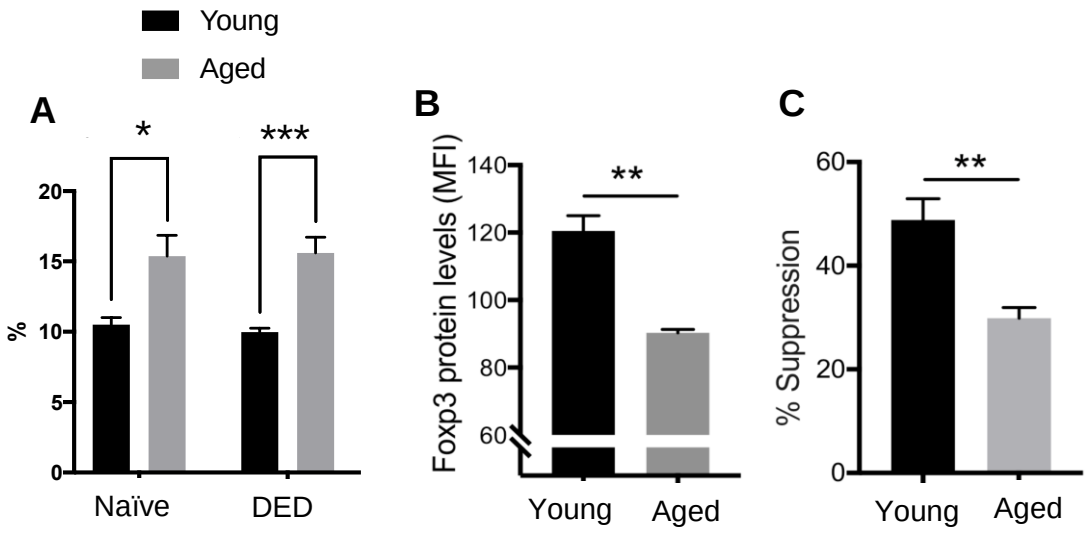

Figure 6. 2 Regulatory T cells isolated from aged mice exposed to two weeks of desiccating stress are greater in frequency, but exhibit decreased Foxp3 expression and suppressive function compared to regulatory T cells derived from young mice

Bar chart depicting frequencies of Tregs in respective groups (A). Foxp3 expression levels (B; mean fluorescent intensity) and suppressive function (C) of young and aged Tregs after 14 days of desiccating stress. n=6/group. Data are depicted as mean +/- SEM. *p<0.05; ***p<0.001.

In light of this preliminary data suggesting age-related functional deficits in Tregs, and the data presented in Chapter 5 showing an increased memory Th17 pool in aged animals, I would like to investigate whether aged Tregs are less effective at suppressing the generation of memory Th17 cells relative to young mice. For example, I might conduct experiments in which Rag1 knockout mice (which have no mature T or B cells) are reconstituted with CD25-depleted $CD4^+$ cells sourced from naïve mice, before being exposed to desiccating stress for 2 weeks. At day 14, the mice would receive adoptive transfer (via tail vein injection) of 100,000 Tregs isolated from either young or aged naïve mice. CFS scores would be evaluated at regulate intervals. A subset of the mice would be euthanized at day 28 for the evaluation of memory Th17 cell frequencies in the draining lymph nodes. The remaining mice would be re-exposed to desiccating stress (i.e. rechallenged) and the recall response would be assessed by evaluating CFS scores and frequencies of effector Th17 cells in the conjunctivae and draining lymph nodes.

References

1. Pascolini D, Mariotti SP. Global estimates of visual impairment: 2010. *Br J Ophthalmol*. 2012;96(5):614-618.
2. Gain P, Jullienne R, He Z, et al. Global Survey of Corneal Transplantation and Eye Banking. *JAMA Ophthalmol*. 2016;134(2):167.
3. Aquavella J V, Qian Y, McCormick GJ, Palakuru JR. Keratoprosthesis. *Cornea*. 2006;25(6):656-662.
4. Salvador-Culla B, Kolovou P. Keratoprosthesis: A Review of Recent Advances in the Field. *J Funct Biomater*. 2016;7(2):13.
5. Buchmann K. Evolution of Innate Immunity: Clues from Invertebrates via Fish to Mammals. *Front Immunol*. 2014;5:459.
6. Riera Romo M, Pérez-Martínez D, Castillo Ferrer C. Innate immunity in vertebrates: an overview. *Immunology*. 2016;148(2):125-139.
7. Tang D, Kang R, Coyne CB, Zeh HJ, Lotze MT. PAMPs and DAMPs: signal 0s that spur autophagy and immunity. *Immunol Rev*. 2012;249(1):158-175.
8. Schleimer RP, Kato A, Kern R, Kuperman D, Avila PC. Epithelium: at the interface of innate and adaptive immune responses. *J Allergy Clin Immunol*. 2007;120(6):1279-1284.
9. Sheridan BS, Lefrançois L. Intraepithelial lymphocytes: to serve and protect. *Curr Gastroenterol Rep*. 2010;12(6):513-521.
10. Olivares-Villagómez D, Van Kaer L. Intestinal Intraepithelial Lymphocytes: Sentinels of the Mucosal Barrier. *Trends Immunol*. 2018;39(4):264-275.
11. McCauley HA, Guasch G. Three cheers for the goblet cell: maintaining homeostasis in mucosal epithelia. *Trends Mol Med*. 2015;21(8):492-503.
12. Corfield AP. Mucins: A biologically relevant glycan barrier in mucosal protection. *Biochim Biophys Acta - Gen Subj*. 2015;1850(1):236-252.
13. Ley K, Hoffman HM, Kubes P, et al. Neutrophils: New insights and open questions. *Sci Immunol*. 2018;3(30):eaat4579.

14. Dale DC, Boxer L, Liles WC. The phagocytes: neutrophils and monocytes. *Blood*. 2008;112(4):935-945.
15. Sadik CD, Kim ND, Luster AD. Neutrophils cascading their way to inflammation. *Trends Immunol*. 2011;32(10):452-460.
16. Gordon S, Plüddemann A. Tissue macrophages: heterogeneity and functions. *BMC Biol*. 2017;15(1):53.
17. Voehringer D. Protective and pathological roles of mast cells and basophils. *Nat Rev Immunol*. 2013;13(5):362-375.
18. Amin K. The role of mast cells in allergic inflammation. *Respir Med*. 2012;106(1):9-14.
19. Galli SJ, Kalesnikoff J, Grimbaldeston MA, Piliponsky AM, Williams CMM, Tsai M. Mast cells as “tunable” effector and immunoregulatory cells: recent advances. *Annu Rev Immunol*. 2005;23(1):749-786.
20. Reber LL, Sibilano R, Mukai K, Galli SJ. Potential effector and immunoregulatory functions of mast cells in mucosal immunity. *Mucosal Immunol*. 2015;8(3):444-463.
21. Gao F, Chiu SM, Motan DAL, et al. Mesenchymal stem cells and immunomodulation: current status and future prospects. *Cell Death Dis*. 2016;7(1):e2062.
22. Mildner A, Jung S. Development and Function of Dendritic Cell Subsets. *Immunity*. 2014;40(5):642-656.
23. Worbs T, Hammerschmidt SI, Förster R. Dendritic cell migration in health and disease. *Nat Rev Immunol*. 2017;17(1):30-48.
24. Clark GJ, Angel N, Kato M, et al. The role of dendritic cells in the innate immune system. *Microbes Infect*. 2000;2(3):257-272.
25. Bonilla FA, Oettgen HC. Adaptive immunity. *J Allergy Clin Immunol*. 2010;125(2):S33-S40.
26. Koch U, Radtke F. Mechanisms of T Cell Development and Transformation.

Annu Rev Cell Dev Biol. 2011;27(1):539-562.

27. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations. *Annu Rev Immunol*. 2010;28:445-489.
28. Ni K, O'Neill H. The role of dendritic cells in T cell activation. *Immunol Cell Biol*. 1997;75(3):223-230.
29. Liew FY. TH1 and TH2 cells: a historical perspective. *Nat Rev Immunol*. 2002;2(1):55-60.
30. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol*. 2004;75(2):163-189.
31. Walker JA, McKenzie ANJ. TH2 cell development and function. *Nat Rev Immunol*. 2017;18(2):121-133.
32. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. *Annu Rev Immunol*. 2009;27(1):485-517.
33. Muranski P, Restifo NP. Essentials of Th17 cell commitment and plasticity. *Blood*. 2013;121(13):2402-2414.
34. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol*. 1995;155(3):1151-1164.
35. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol*. 2003;4(4):330-336.
36. Hori S, Nomura T, Sakaguchi S. Control of Regulatory T Cell Development by the Transcription Factor Foxp3. *Science (80-)*. 2003;299(5609):1057-1061.
37. Yagi H, Nomura T, Nakamura K, et al. Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells. *Int Immunol*. 2004;16(11):1643-1656.
38. Roncador G, Brown PJ, Maestre L, et al. Analysis of FOXP3 protein expression in human CD4+CD25+ regulatory T cells at the single-cell level. *Eur J Immunol*.

2005;35(6):1681-1691.

39. Vignali DAA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol*. 2008;8(7):523-532.

40. Bennett CL, Christie J, Ramsdell F, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet*. 2001;27(1):20-21.

41. Shevach EM, Thornton AM. tTregs, pTregs, and iTregs: similarities and differences. *Immunol Rev*. 2014;259(1):88-102.

42. Foulsham W, Marmalidou A, Amouzegar A, Coco G, Chen Y, Dana R. Review: The function of regulatory T cells at the ocular surface. *Ocul Surf*. 2017;15(4):652-659.

43. Noack M, Miossec P. Th17 and regulatory T cell balance in autoimmune and inflammatory diseases. *Autoimmun Rev*. 2014;13(6):668-677.

44. Zhou L, Lopes JE, Chong MMW, et al. TGF-β-induced Foxp3 inhibits TH17 cell differentiation by antagonizing RORγt function. *Nature*. 2008;453(7192):236-240.

45. Kleinewietfeld M, Hafler DA. The plasticity of human Treg and Th17 cells and its role in autoimmunity. *Semin Immunol*. 2013;25(4):305-312.

46. Yang XO, Nurieva R, Martinez GJ, et al. Molecular Antagonism and Plasticity of Regulatory and Inflammatory T Cell Programs. *Immunity*. 2008;29(1):44-56.

47. Mitchell P, Afzali B, Lombardi G, Lechler RI. The T helper 17–regulatory T cell axis in transplant rejection and tolerance. *Curr Opin Organ Transplant*. 2009;14(4):326-331.

48. Galletti JG, Guzmán M, Giordano MN. Mucosal immune tolerance at the ocular surface in health and disease. *Immunology*. 2017;150(4):397-407.

49. Meek KM, Knupp C. Corneal structure and transparency. *Prog Retin Eye Res*. 2015;49:1-16.

50. Foulsham W, Coco G, Amouzegar A, Chauhan SK, Dana R. When Clarity Is

Crucial: Regulating Ocular Surface Immunity. *Trends Immunol*. 2018;39(4):288-301.

51. Beebe DC. Maintaining transparency: a review of the developmental physiology and pathophysiology of two avascular tissues. *Semin Cell Dev Biol*. 2008;19(2):125-133.
52. Yang AY, Chow J, Liu J. Corneal Innervation and Sensation: The Eye and Beyond. *Yale J Biol Med*. 2018;91(1):13-21.
53. Bowling B. *Kanski's Clinical Ophthalmology*. 8th Ed. Amsterdam: Elsevier; 2016.
54. Forrester J V., Dick A, McMenamin P, Lee W. *The Eye: Basic Sciences in Practice*. London: Saunders; 1999.
55. Ordonez P, Di Girolamo N. Limbal Epithelial Stem Cells: Role of the Niche Microenvironment. *Stem Cells*. 2012;30(2):100-107.
56. Wilson SE, Hong JW. Bowman's layer structure and function: critical or dispensable to corneal function? A hypothesis. *Cornea*. 2000;19(4):417-420.
57. Hoffer KJ, Kraff MC. Normal Endothelial Cell Count Range. *Ophthalmology*. 1980;87(9):861-866.
58. Ramos T, Scott D, Ahmad S. An Update on Ocular Surface Epithelial Stem Cells: Cornea and Conjunctiva. *Stem Cells Int*. 2015;2015:601731.
59. Conrady CD, Joos ZP, Patel BCK. Review: The Lacrimal Gland and Its Role in Dry Eye. *J Ophthalmol*. 2016;2016:1-11.
60. Knop N, Knop E. Conjunctiva-associated lymphoid tissue in the human eye. *Invest Ophthalmol Vis Sci*. 2000;41(6):1270-1279.
61. Holly FJ, Lemp MA. Tear physiology and dry eyes. *Surv Ophthalmol*. 22(2):69-87.
62. Willcox MDP, Argüeso P, Georgiev GA, et al. TFOS DEWS II Tear Film Report. *Ocul Surf*. 2017;15(3):366-403.
63. Prydal JI, Artal P, Woon H, Campbell FW. Study of human precorneal tear film

thickness and structure using laser interferometry. *Invest Ophthalmol Vis Sci*. 1992;33(6):2006-2011.

64. Benhar I, London A, Schwartz M. The privileged immunity of immune privileged organs: the case of the eye. *Front Immunol*. 2012;3:296.

65. Medawar PB. 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):58-69.

66. El Annan J, Goyal S, Zhang Q, Freeman GJ, Sharpe AH, Dana R. Regulation of T-cell chemotaxis by programmed death-ligand 1 (PD-L1) in dry eye-associated corneal inflammation. *Invest Ophthalmol Vis Sci*. 2010;51(7):3418-3423.

67. Morris JE, Zobell S, Yin X-T, et al. Mice with mutations in Fas and Fas ligand demonstrate increased herpetic stromal keratitis following corneal infection with HSV-1. *J Immunol*. 2012;188(2):793-799.

68. Ferrari G, Hajrasouliha AR, Sadrai Z, Ueno H, Chauhan SK, Dana R. Nerves and neovessels inhibit each other in the cornea. *Invest Ophthalmol Vis Sci*. 2013;54(1):813-820.

69. Ambati BK, Nozaki M, Singh N, et al. Corneal avascularity is due to soluble VEGF receptor-1. *Nature*. 2006;443(7114):993-997.

70. Cursiefen C, Chen L, Saint-Geniez M, et al. Nonvascular VEGF receptor 3 expression by corneal epithelium maintains avascularity and vision. *Proc Natl Acad Sci*. 2006;103(30):11405-11410.

71. Contreras-Ruiz L, Masli S. Immunomodulatory cross-talk between conjunctival goblet cells and dendritic cells. Wallace GR, ed. *PLoS One*. 2015;10(3):e0120284.

72. Ma P, Bian F, Wang Z, et al. Human corneal epithelium-derived thymic stromal lymphopoietin links the innate and adaptive immune responses via TLRs and Th2 cytokines. *Invest Ophthalmol Vis Sci*. 2009;50(6):2702-2709.

73. McDermott AM. Antimicrobial compounds in tears. *Exp Eye Res*. 2013;117:53-

61.

74. Kugadas A, Gadjeva M. Impact of Microbiome on Ocular Health. *Ocul Surf*. 2016;14(3):342-349.

75. Ablamowicz AF, Nichols JJ. Ocular Surface Membrane-Associated Mucins. *Ocul Surf*. 2016;14(3):331-341.

76. Cousins SW, McCabe MM, Danielpour D, Streilein JW. Identification of transforming growth factor-beta as an immunosuppressive factor in aqueous humor. *Invest Ophthalmol Vis Sci*. 1991;32(8):2201-2211.

77. Chen C, Michelini-Norris B, Stevens S, et al. Measurement of mRNAs for TGFss and extracellular matrix proteins in corneas of rats after PRK. *Invest Ophthalmol Vis Sci*. 2000;41(13):4108-4116.

78. Dana MR, Yamada J, Streilein JW. Topical interleukin 1 receptor antagonist promotes corneal transplant survival. *Transplantation*. 1997;63(10):1501-1507.

79. Amouzegar A, Chauhan SK, Dana R. Alloimmunity and Tolerance in Corneal Transplantation. *J Immunol*. 2016;196(10):3983-3991.

80. Tran MT, Lausch RN, Oakes JE. Substance P differentially stimulates IL-8 synthesis in human corneal epithelial cells. *Invest Ophthalmol Vis Sci*. 2000;41(12):3871-3877.

81. Mashaghi A, Marmalidou A, Tehrani M, Grace PM, Pothoulakis C, Dana R. Neuropeptide substance P and the immune response. *Cell Mol Life Sci*. 2016;73(22):4249-4264.

82. Sabatino F, Di Zazzo A, De Simone L, Bonini S. The Intriguing Role of Neuropeptides at the Ocular Surface. *Ocul Surf*. 2017;15(1):2-14.

83. Taylor AW, Lee DJ. The Alpha-Melanocyte Stimulating Hormone Induces Conversion of Effector T cells into Treg cells. *J Transplant*. 2011;2011:246856.

84. Adams RH, Alitalo K. Molecular regulation of angiogenesis and lymphangiogenesis. *Nat Rev Mol Cell Biol*. 2007;8(6):464-478.

85. Albuquerque RJC, Hayashi T, Cho WG, et al. Alternatively spliced vascular

endothelial growth factor receptor-2 is an essential endogenous inhibitor of lymphatic vessel growth. *Nat Med*. 2009;15(9):1023-1030.

86. Yamagami S, Dana MR, Tsuru T. Draining lymph nodes play an essential role in alloimmunity generated in response to high-risk corneal transplantation. *Cornea*. 2002;21(4):405-409.

87. Hos D, Bock F, Bachmann B, Cursiefen C. Corneal Neovascular Diseases. In: Springer International Publishing; 2016:159-172.

88. Mastropasqua L, Nubile M, Lanzini M, et al. Epithelial dendritic cell distribution in normal and inflamed human cornea: in vivo confocal microscopy study. *Am J Ophthalmol*. 2006;142(5):736-744.

89. Forrester J V., Xu H, Kuffová L, Dick AD, McMenamin PG. Dendritic cell physiology and function in the eye. *Immunol Rev*. 2010;234(1):282-304.

90. Pearlman E, Johnson A, Adhikary G, et al. Toll-like receptors at the ocular surface. *Ocul Surf*. 2008;6(3):108-116.

91. Yamagami S, Yokoo S, Usui T, Yamagami H, Amano S, Ebihara N. Distinct Populations of Dendritic Cells in the Normal Human Donor Corneal Epithelium. *Investig Opthalmology Vis Sci*. 2005;46(12):4489.

92. Hattori T, Takahashi H, Dana R. Novel Insights Into the Immunoregulatory Function and Localization of Dendritic Cells. *Cornea*. 2016;35:S49-S54.

93. Mayer WJ, Irschick UM, Moser P, et al. Characterization of antigen-presenting cells in fresh and cultured human corneas using novel dendritic cell markers. *Invest Ophthalmol Vis Sci*. 2007;48(10):4459-4467.

94. Hamrah P, Zhang Q, Liu Y, Dana MR. Novel characterization of MHC class II-negative population of resident corneal Langerhans cell-type dendritic cells. *Invest Ophthalmol Vis Sci*. 2002;43(3):639-646.

95. Hattori T, Chauhan SK, Lee H, et al. Characterization of Langerin-expressing dendritic cell subsets in the normal cornea. *Invest Ophthalmol Vis Sci*. 2011;52(7):4598-4604.

96. Hamrah P, Liu Y, Zhang Q, Dana MR. The corneal stroma is endowed with a significant number of resident dendritic cells. *Invest Ophthalmol Vis Sci*. 2003;44(2):581-589.

97. Huq S, Liu Y, Benichou G, Dana MR. Relevance of the direct pathway of sensitization in corneal transplantation is dictated by the graft bed microenvironment. *J Immunol*. 2004;173(7):4464-4469.

98. Khandelwal P, Blanco-Mezquita T, Emami P, et al. Ocular mucosal CD11b+ and CD103+ mouse dendritic cells under normal conditions and in allergic immune responses. Kovats S, ed. *PLoS One*. 2013;8(5):e64193.

99. Hattori T, Saban DR, Emami-naeini P, et al. Donor-derived, tolerogenic dendritic cells suppress immune rejection in the indirect allosensitization-dominant setting of corneal transplantation. *J Leukoc Biol*. 2012;91(4):621-627.

100. Paunicka KJ, Mellon J, Robertson D, Petroll M, Brown JR, Niederkorn JY. Severing Corneal Nerves in One Eye Induces Sympathetic Loss of Immune Privilege and Promotes Rejection of Future Corneal Allografts Placed in Either Eye. *Am J Transplant*. 2015;15(6):1490-1501.

101. Gaddipati S, Rao P, Jerome AD, Burugula BB, Gerard NP, Suvas S. Loss of Neurokinin-1 Receptor Alters Ocular Surface Homeostasis and Promotes an Early Development of Herpes Stromal Keratitis. *J Immunol*. 2016;197(10):4021-4033.

102. Springer J, Geppetti P, Fischer A, Groneberg DA. Calcitonin gene-related peptide as inflammatory mediator. *Pulm Pharmacol Ther*. 2003;16(3):121-130.

103. Dimitrijević M, Stanojević S. The intriguing mission of neuropeptide Y in the immune system. *Amino Acids*. 2013;45(1):41-53.

104. Phan TA, Taylor AW. The neuropeptides α-MSH and NPY modulate phagocytosis and phagolysosome activation in RAW 264.7 cells. *J Neuroimmunol*. 2013;260(1-2):9-16.

105. Delgado M, Ganea D. Vasoactive intestinal peptide: a neuropeptide with

pleiotropic immune functions. *Amino Acids*. 2013;45(1):25-39.

106. Zirm EK. Eine erfolgreiche totale Keratoplastik (A successful total keratoplasty). 1906. *Refract Corneal Surg*. 5(4):258-261.

107. Tan DTH, Dart JKG, Holland EJ, Kinoshita S. Corneal transplantation. *Lancet (London, England)*. 2012;379(9827):1749-1761.

108. Anshu A, Price MO, Price FW. Risk of Corneal Transplant Rejection Significantly Reduced with Descemet's Membrane Endothelial Keratoplasty. *Ophthalmology*. 2012;119(3):536-540.

109. Dana MR, Qian Y, Hamrah P. Twenty-five-year panorama of corneal immunology: emerging concepts in the immunopathogenesis of microbial keratitis, peripheral ulcerative keratitis, and corneal transplant rejection. *Cornea*. 2000;19(5):625-643.

110. Price FW, Whitson WE, Collins KS, Marks RG. Five-year corneal graft survival. A large, single-center patient cohort. *Arch Ophthalmol (Chicago, Ill 1960)*. 1993;111(6):799-805.

111. The Collaborative Corneal Transplantation Studies Research Group. The collaborative corneal transplantation studies (CCTS): effectiveness of histocompatibility matching in high-risk corneal transplantation. *Arch Ophthalmol*. 1992;110(10):1392.

112. Coster DJ, Williams KA. The impact of corneal allograft rejection on the long-term outcome of corneal transplantation. *Am J Ophthalmol*. 2005;140(6):1112-1122.

113. Khodadoust AA, Silverstein AM. Transplantation and rejection of individual cell layers of the cornea. *Invest Ophthalmol*. 1969;8(2):180-195.

114. Guilbert E, Bullet J, Sandali O, Basli E, Laroche L, Borderie VM. Long-term rejection incidence and reversibility after penetrating and lamellar keratoplasty. *Am J Ophthalmol*. 2013;155(3):560-569.e2.

115. Panda A, Vanathi M, Kumar A, Dash Y, Priya S. Corneal Graft Rejection. *Surv*

Ophthalmol. 2007;52(4):375-396.

116. Hamrah P, Liu Y, Zhang Q, et al. Alterations in corneal stromal dendritic cell phenotype and distribution in inflammation. 2003;121(8).
117. Zhu S, Dekaris I, Duncker G, Dana MR. Early Expression of Proinflammatory Cytokines Interleukin-1 and Tumor Necrosis Factor-alpha after Corneal Transplantation. *J Interf Cytokine Res*. 1999;19(6):661-669.
118. Yamagami S, Hamrah P, Zhang Q, Liu Y, Huq S, Dana MR. Early ocular chemokine gene expression and leukocyte infiltration after high-risk corneal transplanation. *Line Mol Vis*. 2005;11:632-640.
119. Zhu SN, Yamada J, Streilein JW, Dana MR. ICAM-1 deficiency suppresses host allosensitization and rejection of MHC-disparate corneal transplants. *Transplantation*. 2000;69(5):1008-1013.
120. Grimaldo S, Yuen D, Ecoiffier T, Chen L. Very Late Antigen-1 Mediates Corneal Lymphangiogenesis. *Investig Opthalmology Vis Sci*. 2011;52(7):4808.
121. Zhu SN, Dana MR. Expression of cell adhesion molecules on limbal and neovascular endothelium in corneal inflammatory neovascularization. *Invest Ophthalmol Vis Sci*. 1999;40(7):1427-1434.
122. McBride BW, McGill JI, Smith JL. MHC class I and class II antigen expression in normal human corneas and in corneas from cases of herpetic keratitis. *Immunology*. 1988;65(4):583-587.
123. Chong E-M, Dana MR. Graft failure IV. Immunologic mechanisms of corneal transplant rejection. *Int Ophthalmol*. 2008;28(3):209-222.
124. Dekaris I, Zhu SN, Dana MR. TNF-alpha regulates corneal Langerhans cell migration. *J Immunol*. 1999;162(7):4235-4239.
125. Sano Y, Streilein JW, Ksander BR. 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):963-970.

126. Liu Y, Hamrah P, Zhang Q, Taylor AW, Dana MR. Draining lymph nodes of corneal transplant hosts exhibit evidence for donor major histocompatibility complex (MHC) class II-positive dendritic cells derived from MHC class II-negative grafts. *J Exp Med*. 2002;195(2):259-268.

127. Bachmann BO, Bock F, Wiegand SJ, et al. Promotion of Graft Survival by Vascular Endothelial Growth Factor A Neutralization After High-Risk Corneal Transplantation. *Arch Ophthalmol*. 2008;126(1):71.

128. Emami-Naeini P, Dohlman TH, Omoto M, et al. Soluble vascular endothelial growth factor receptor-3 suppresses allosensitization and promotes corneal allograft survival. *Graefe's Arch Clin Exp Ophthalmol*. 2014;252(11):1755-1762.

129. Dohlman TH, Omoto M, Hua J, et al. VEGF-trap Aflibercept Significantly Improves Long-term Graft Survival in High-risk Corneal Transplantation. *Transplantation*. 2015;99(4):678-686.

130. Chauhan SK, Dohlman TH, Dana R. Corneal Lymphatics: Role in Ocular Inflammation as Inducer and Responder of Adaptive Immunity. *J Clin Cell Immunol*. 2014;5(05).

131. Goldberg MF, Ferguson TA, Pepose JS. Detection of cellular adhesion molecules in inflamed human corneas. *Ophthalmology*. 1994;101(1):161-168.

132. Hong JW, Liu JJ, Lee JS, et al. Proinflammatory chemokine induction in keratocytes and inflammatory cell infiltration into the cornea. *Invest Ophthalmol Vis Sci*. 2001;42(12):2795-2803.

133. Spandau UHM, Toksoy A, Verhaart S, Gillitzer R, Kruse FE. High Expression of Chemokines Gro-α (CXCL-1), IL-8 (CXCL-8), and MCP-1 (CCL-2) in Inflamed Human Corneas In Vivo. *Arch Ophthalmol*. 2003;121(6):825.

134. Chen L, Hamrah P, Cursiefen C, et al. Vascular endothelial growth factor receptor-3 mediates induction of corneal alloimmunity. *Nat Med*. 2004;10:2187-2193.

135. Jin Y, Shen L, Chong E-M, et al. The chemokine receptor CCR7 mediates

corneal antigen-presenting cell trafficking. *Mol Vis*. 2007;13:626-634.

136. Johnson LA, Jackson DG. Inflammation-induced secretion of CCL21 in lymphatic endothelium is a key regulator of integrin-mediated dendritic cell transmigration. *Int Immunol*. 2010;22(10):839-849.

137. Hegde S, Beauregard C, Mayhew E, Niederkorn JY. CD4(+) T-cell-mediated mechanisms of corneal allograft rejection: role of Fas-induced apoptosis. *Transplantation*. 2005;79(1):23-31.

138. Yamada J, Kurimoto I, Streilein JW. Role of CD4+ T cells in immunobiology of orthotopic corneal transplants in mice. *Invest Ophthalmol Vis Sci*. 1999;40(11):2614-2621.

139. He YG, Ross J, Niederkorn JY. Promotion of murine orthotopic corneal allograft survival by systemic administration of anti-CD4 monoclonal antibody. *Invest Ophthalmol Vis Sci*. 1991;32(10):2723-2728.

140. Sano Y, Osawa H, Sotozono C, Kinoshita S. Cytokine expression during orthotopic corneal allograft rejection in mice. *Invest Ophthalmol Vis Sci*. 1998;39(10):1953-1957.

141. Yamada J, Hamuro J, Fukushima A, et al. MHC-Matched Corneal Allograft Rejection in an IFN-γ/IL-17–Independent Manner in C57BL/6 Mice. *Investig Opthalmology Vis Sci*. 2009;50(5):2139.

142. Chen H, Wang W, Xie H, et al. A pathogenic role of IL- 17 at the early stage of corneal allograft rejection. *Transpl Immunol*. 2009;21(3):155-161.

143. Chauhan SK, Jin Y, Goyal S, et al. A novel pro-lymphangiogenic function for Th17/IL-17. *Blood*. 2011;118(17):4630-4634.

144. Cunnusamy K, Chen PW, Niederkorn JY. IL-17 promotes immune privilege of corneal allografts. *J Immunol*. 2010;185(8):4651-4658.

145. Cunnusamy K, Chen PW, Niederkorn JY. IL-17A-Dependent CD4+CD25+ Regulatory T Cells Promote Immune Privilege of Corneal Allografts. *J Immunol*. 2011;186(12):6737-6745.

146. Ksander BR, Sano Y, Streilein JW. Role of donor-specific cytotoxic T cells in rejection of corneal allografts in normal and high-risk eyes. *Transpl Immunol*. 1996;4(1):49-52.

147. Minamoto A, Sakata H, Tsumura K, Yamamoto M, Kusunoki S. Induction of Cytotoxic T Lymphocytes from Splenocytes after Orthotopic Penetrating Keratoplasty in the Rat. *Ophthalmologica*. 1994;208(2):105-109.

148. Van der Veen G, Broersma L, Van Rooijen N, Van Rij G, Van der Gaag R. Cytotoxic T lymphocytes and antibodies after orthotropic penetrating keratoplasty in rats treated with dichloromethylene diphosphonate encapsulated liposomes. *Curr Eye Res*. 1998;17(10):1018-1026.

149. Callanan D, Peeler J, Niederkorn JY. Characteristics of rejection of orthotopic corneal allografts in the rat. *Transplantation*. 1988;45(2):437-443.

150. Niederkorn JY, Stevens C, Mellon J, Mayhew E. Differential roles of CD8+ and CD8- T lymphocytes in corneal allograft rejection in “high-risk” hosts. *Am J Transpl*. 2006;6(4):705-713.

151. Yamada J, Ksander BR, Streilein JW. Cytotoxic T cells play no essential role in acute rejection of orthotopic corneal allografts in mice. *Invest Ophthalmol Vis Sci*. 2001;42(2):386-392.

152. Boisgérault F, Liu Y, Anosova N, et al. Role of CD4+ and CD8+ T cells in allorecognition: lessons from corneal transplantation. *J Immunol*. 2001;167(4):1891-1899.

153. Wood KJ, Sakaguchi S. Regulatory Lymphocytes: Regulatory T cells in transplantation tolerance. *Nat Rev Immunol*. 2003;3(3):199-210.

154. Walsh PT, Taylor DK, Turka LA. Tregs and transplantation tolerance. *J Clin Invest*. 2004;114(10):1398-1403.

155. Chauhan SK, Saban DR, Lee HK, Dana R. Levels of Foxp3 in regulatory T cells reflect their functional status in transplantation. *J Immunol*. 2009;182(1):148-153.

156. Cunnusamy K, Chen PW, Niederkorn JY. Paradigm shifts in the role of CD4+ T cells in keratoplasty. *Discov Med*. 2010;10(54):452-461.

157. Skelsey ME, Mellon J, Niederkorn JY. Gamma delta T cells are needed for ocular immune privilege and corneal graft survival. *J Immunol*. 2001;166(7):4327-4333.

158. LI B, TIAN L, DIAO Y, LI X, ZHAO L, WANG X. Exogenous IL-10 induces corneal transplantation immune tolerance by a mechanism associated with the altered Th1/Th2 cytokine ratio and the increased expression of TGF-β. *Mol Med Rep*. 2014;9(6):2245-2250.

159. Inomata T, Hua J, Di Zazzo A, Dana R. Impaired Function of Peripherally Induced Regulatory T Cells in Hosts at High Risk of Graft Rejection. *Sci Rep*. 2016;6(1):39924.

160. DuPage M, Bluestone JA. Harnessing the plasticity of CD4(+) T cells to treat immune-mediated disease. *Nat Rev Immunol*. 2016;16(3):149-163.

161. Sawant D V., Vignali DAA. Once a Treg, always a Treg? 2014;259(1).

162. Hua J, Inomata T, Chen Y, et al. Pathological conversion of regulatory T cells is associated with loss of allotolerance. *Sci Rep*. 2018;8(1):7059.

163. Chauhan SK, Saban DR, Dohlman TH, Dana R. CCL-21 conditioned regulatory T cells induce allotolerance through enhanced homing to lymphoid tissue. *J Immunol*. 2014;192(2):817-823.

164. Shao C, Chen Y, Nakao T, et al. Local Delivery of Regulatory T Cells Promotes Corneal Allograft Survival. *Transplantation*. September 2018.

165. Tahvildari M, Omoto M, Chen Y, et al. In Vivo Expansion of Regulatory T Cells by Low-Dose Interleukin-2 Treatment Increases Allograft Survival in Corneal Transplantation. *Transplantation*. 2016;100(3).

166. Turnquist HR, Raimondi G, Zahorchak AF, Fischer RT, Wang Z, Thomson AW. Rapamycin-conditioned dendritic cells are poor stimulators of allogeneic CD4+ T cells, but enrich for antigen-specific Foxp3+ T regulatory cells and promote

organ transplant tolerance. *J Immunol*. 2007;178(11):7018-7031.

167. Yan F, Cai L, Hui Y, chen S, Meng H, Huang Z. Tolerogenic dendritic cells suppress murine corneal allograft rejection by modulating CD28/CTLA-4 expression on regulatory T cells. *Cell Biol Int*. 2014;38(7):835-848.
168. Tahvildari M, Emami-Naeini P, Omoto M, Mashaghi A, Chauhan SK, Dana R. Treatment of donor corneal tissue with immunomodulatory cytokines: a novel strategy to promote graft survival in high-risk corneal transplantation. *Sci Rep*. 2017;7(1):971.
169. Ma S, Xie N, Li W, Yuan B, Shi Y, Wang Y. Immunobiology of mesenchymal stem cells. *Cell Death Differ*. 2014;21(2):216-225.
170. Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. *J Cell Physiol*. 2007;213(2):341-347.
171. Sahu A, Foulsham W, Amouzegar A, Mittal SK, Chauhan SK. The therapeutic application of mesenchymal stem cells at the ocular surface. *Ocul Surf*. January 2019.
172. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. *Science*. 1999;284(5411):143-147.
173. Fernandes KJL, McKenzie IA, Mill P, et al. A dermal niche for multipotent adult skin-derived precursor cells. *Nat Cell Biol*. 2004;6(11):1082-1093.
174. Jiang Y, Jahagirdar BN, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature*. 2002;418(6893):41-49.
175. Sasaki M, Abe R, Fujita Y, Ando S, Inokuma D, Shimizu H. Mesenchymal stem cells are recruited into wounded skin and contribute to wound repair by transdifferentiation into multiple skin cell type. *J Immunol*. 2008;180(4):2581-2587.
176. Sanchez-Ramos J, Song S, Cardozo-Pelaez F, et al. Adult Bone Marrow Stromal Cells Differentiate into Neural Cells in Vitro. *Exp Neurol*. 2000;164(2):247-256.

177. Shi Y, Su J, Roberts AI, Shou P, Rabson AB, Ren G. How mesenchymal stem cells interact with tissue immune responses. *Trends Immunol*. 2012;33(3):136-143.

178. Knaän-Shanzer S. Concise Review: The Immune Status of Mesenchymal Stem Cells and Its Relevance for Therapeutic Application. *Stem Cells*. 2014;32(3):603-608.

179. Devine SM, Cobbs C, Jennings M, Bartholomew A, Hoffman R. Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. *Blood*. 2003;101(8):2999-3001.

180. Kawada H, Fujita J, Kinjo K, et al. Nonhematopoietic mesenchymal stem cells can be mobilized and differentiate into cardiomyocytes after myocardial infarction. *Blood*. 2004;104(12):3581-3587.

181. Ren G, Su J, Zhang L, et al. Species Variation in the Mechanisms of Mesenchymal Stem Cell-Mediated Immunosuppression. *Stem Cells*. 2009;27(8):1954-1962.

182. Ren G, Zhang L, Zhao X, et al. Mesenchymal Stem Cell-Mediated Immunosuppression Occurs via Concerted Action of Chemokines and Nitric Oxide. *Cell Stem Cell*. 2008;2(2):141-150.

183. Ortiz LA, DuTreil M, Fattman C, et al. Interleukin 1 receptor antagonist mediates the antiinflammatory and antifibrotic effect of mesenchymal stem cells during lung injury. *Proc Natl Acad Sci*. 2007;104(26):11002-11007.

184. Németh K, Leelahavanichkul A, Yuen PST, et al. Bone marrow stromal cells attenuate sepsis via prostaglandin E2–dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med*. 2009;15(1):42-49.

185. Lee RH, Pulin AA, Seo MJ, et al. Intravenous hMSCs Improve Myocardial Infarction in Mice because Cells Embolized in Lung Are Activated to Secrete the Anti-inflammatory Protein TSG-6. *Cell Stem Cell*. 2009;5(1):54-63.

186. Nemeth K, Keane-Myers A, Brown JM, et al. Bone marrow stromal cells use TGF-β to suppress allergic responses in a mouse model of ragweed-induced asthma. *Proc Natl Acad Sci*. 2010;107(12):5652-5657.

187. DelaRosa O, Lombardo E, Beraza A, et al. Requirement of IFN-γ-mediated indoleamine 2,3-dioxygenase expression in the modulation of lymphocyte proliferation by human adipose-derived stem cells. *Tissue Eng Part A*. 2009;15(10):2795-2806.

188. Spaggiari GM, Abdelrazik H, Becchetti F, Moretta L. MSCs inhibit monocyte-derived DC maturation and function by selectively interfering with the generation of immature DCs: central role of MSC-derived prostaglandin E2. *Blood*. 2009;113(26):6576-6583.

189. Ren G, Zhao X, Zhang L, et al. Inflammatory Cytokine-Induced Intercellular Adhesion Molecule-1 and Vascular Cell Adhesion Molecule-1 in Mesenchymal Stem Cells Are Critical for Immunosuppression. *J Immunol*. 2010;184(5):2321-2328.

190. Omoto M, Katikireddy KR, Rezazadeh A, Dohlman TH, Chauhan SK. Mesenchymal stem cells home to inflamed ocular surface and suppress allosensitization in corneal transplantation. *Invest Ophthalmol Vis Sci*. 2014;55(10):6631-6638.

191. Lan Y, Kodati S, Lee HS, Omoto M, Jin Y, Chauhan SK. Kinetics and function of mesenchymal stem cells in corneal injury. *Invest Ophthalmol Vis Sci*. 2012;53(7):3638-3644.

192. Oh JY, Lee RH, Yu JM, et al. Intravenous mesenchymal stem cells prevented rejection of allogeneic corneal transplants by aborting the early inflammatory response. *Mol Ther*. 2012;20(11):2143-2152.

193. Jia Z, Jiao C, Zhao S, et al. Immunomodulatory effects of mesenchymal stem cells in a rat corneal allograft rejection model. *Exp Eye Res*. 2012;102:44-49.

194. Treacy O, O'Flynn L, Ryan AE, et al. Mesenchymal stem cell therapy promotes

corneal allograft survival in rats by local and systemic immunomodulation. *Am J Transplant*. 2014;14(9):2023-2036.

195. Molnarfi N, Benkhoucha M, Funakoshi H, Nakamura T, Lalive PH. Hepatocyte growth factor: A regulator of inflammation and autoimmunity. *Autoimmun Rev*. 2015;14(4):293-303.
196. Ilangumaran S, Villalobos-Hernandez A, Bobbala D, Ramanathan S. The hepatocyte growth factor (HGF)–MET receptor tyrosine kinase signaling pathway: Diverse roles in modulating immune cell functions. *Cytokine*. 2016;82:125-139.
197. Nakamura T, Sakai K, Nakamura T, Matsumoto K. Hepatocyte growth factor twenty years on: Much more than a growth factor. *J Gastroenterol Hepatol*. 2011;26:188-202.
198. Kennelly H, Mahon BP, English K. Human mesenchymal stromal cells exert HGF dependent cytoprotective effects in a human relevant pre-clinical model of COPD. *Sci Rep*. 2016;6:38207.
199. Mittal SK, Omoto M, Amouzegar A, et al. Restoration of Corneal Transparency by Mesenchymal Stem Cells. *Stem Cell Reports*. 2016;7(4):583-590.
200. Omoto M, Suri K, Amouzegar A, et al. Hepatocyte Growth Factor Suppresses Inflammation and Promotes Epithelium Repair in Corneal Injury. *Mol Ther*. 2017;25(8):1881-1888.
201. Cahill EF, Kennelly H, Carty F, Mahon BP, English K. Hepatocyte Growth Factor Is Required for Mesenchymal Stromal Cell Protection Against Bleomycin-Induced Pulmonary Fibrosis. *Stem Cells Transl Med*. 2016;5(10):1307-1318.
202. Craig JP, Nelson JD, Azar DT, et al. TFOS DEWS II Report Executive Summary. *Ocul Surf*. 2017;15(4):802-812.
203. Miljanović B, Dana R, Sullivan DA, Schaumberg DA. Impact of dry eye syndrome on vision-related quality of life. *Am J Ophthalmol*. 2007;143(3):409-415.

204. Crnej A, Kheirkhah A, Ren A, et al. Patients' Perspectives on Their Dry Eye Disease. *Ocul Surf*. 2016;14(4):440-446.

205. Bradley JL, Özer Stillman I, Pivneva I, Guerin A, Evans AM, Dana R. Dry eye disease ranking among common reasons for seeking eye care in a large US claims database. *Clin Ophthalmol*. 2019;Volume 13:225-232.

206. Dana R, Bradley JL, Guerin A, et al. Estimated Prevalence and Incidence of Dry Eye Disease Based on Coding Analysis of a Large, All-age United States Health Care System. *Am J Ophthalmol*. February 2019.

207. Schein OD, Muñoz B, Tielsch JM, Bandeen-Roche K, West S. Prevalence of dry eye among the elderly. *Am J Ophthalmol*. 1997;124(6):723-728.

208. Moss SE, Klein R, Klein BE. Prevalence of and risk factors for dry eye syndrome. *Arch Ophthalmol (Chicago, Ill 1960)*. 2000;118(9):1264-1268.

209. Stapleton F, Alves M, Bunya VY, et al. TFOS DEWS II Epidemiology Report. *Ocul Surf*. 2017;15(3):334-365.

210. Bron AJ, de Paiva CS, Chauhan SK, et al. TFOS DEWS II pathophysiology report. *Ocul Surf*. 2017;15(3):438-510.

211. Stevenson W, Chauhan SK, Dana R. Dry Eye Disease. *Arch Ophthalmol*. 2012;130(1):90.

212. Barabino S, Chen Y, Chauhan S, Dana R. Ocular surface immunity: homeostatic mechanisms and their disruption in dry eye disease. *Prog Retin Eye Res*. 2012;31(3):271-285.

213. Luo L, Li D-Q, Corrales RM, Pflugfelder SC. Hyperosmolar saline is a proinflammatory stress on the mouse ocular surface. *Eye Contact Lens*. 2005;31(5):186-193.

214. De Paiva CS, Pangelinan SB, Chang E, et al. Essential Role for c-Jun N-Terminal Kinase 2 in Corneal Epithelial Response to Desiccating Stress. *Arch Ophthalmol*. 2009;127(12):1625.

215. Li D-Q, Luo L, Chen Z, Kim H-S, Song XJ, Pflugfelder SC. JNK and ERK MAP

kinases mediate induction of IL-1β, TNF-α and IL-8 following hyperosmolar stress in human limbal epithelial cells. *Exp Eye Res*. 2006;82(4):588-596.

216. Li D-Q, Chen Z, Song XJ, Luo L, Pflugfelder SC. Stimulation of Matrix Metalloproteinases by Hyperosmolarity via a JNK Pathway in Human Corneal Epithelial Cells. *Investig Opthalmology Vis Sci*. 2004;45(12):4302.
217. Chotikavanich S, de Paiva CS, Li DQ, et al. Production and Activity of Matrix Metalloproteinase-9 on the Ocular Surface Increase in Dysfunctional Tear Syndrome. *Investig Opthalmology Vis Sci*. 2009;50(7):3203.
218. Pflugfelder SC, Farley W, Luo L, et al. Matrix Metalloproteinase-9 Knockout Confers Resistance to Corneal Epithelial Barrier Disruption in Experimental Dry Eye. *Am J Pathol*. 2005;166(1):61-71.
219. Solomon A, Dursun D, Liu Z, Xie Y, Macri A, Pflugfelder SC. Pro- and anti-inflammatory forms of interleukin-1 in the tear fluid and conjunctiva of patients with dry-eye disease. *Invest Ophthalmol Vis Sci*. 2001;42(10):2283-2292.
220. Corrales RM, Stern ME, De Paiva CS, Welch J, Li D-Q, Pflugfelder SC. Desiccating stress stimulates expression of matrix metalloproteinases by the corneal epithelium. *Invest Ophthalmol Vis Sci*. 2006;47(8):3293-3302.
221. Beardsley RM, De Paiva CS, Power DF, Pflugfelder SC. Desiccating Stress Decreases Apical Corneal Epithelial Cell Size-Modulation by the Metalloproteinase Inhibitor Doxycycline. *Cornea*. 2008;27(8):935-940.
222. De Paiva CS, Corrales RM, Villarreal AL, et al. Corticosteroid and doxycycline suppress MMP-9 and inflammatory cytokine expression, MAPK activation in the corneal epithelium in experimental dry eye. *Exp Eye Res*. 2006;83(3):526-535.
223. Narayanan S, Corrales RM, Farley W, McDermott AM, Pflugfelder SC. Interleukin-1 Receptor-1-deficient Mice Show Attenuated Production of Ocular Surface Inflammatory Cytokines in Experimental Dry Eye. *Cornea*. 2008;27(7):811-817.
224. Lam H, Bleiden L, de Paiva CS, Farley W, Stern ME, Pflugfelder SC. Tear

cytokine profiles in dysfunctional tear syndrome. *Am J Ophthalmol*. 2009;147(2):198-205. e1.

225. Massingale ML, Li X, Vallabhajosyula M, Chen D, Wei Y, Asbell PA. Analysis of Inflammatory Cytokines in the Tears of Dry Eye Patients. *Cornea*. 2009;28(9):1023-1027.
226. Enríquez-de-Salamanca A, Castellanos E, Stern ME, et al. Tear cytokine and chemokine analysis and clinical correlations in evaporative-type dry eye disease. *Mol Vis*. 2010;16:862-873.
227. Pflugfelder SC, Jones D, Ji Z, Afonso A, Monroy D. Altered cytokine balance in the tear fluid and conjunctiva of patients with Sjögren's syndrome keratoconjunctivitis sicca. *Curr Eye Res*. 1999;19(3):201-211.
228. Robertson MJ. Role of chemokines in the biology of natural killer cells. *J Leukoc Biol*. 2002;71(2):173-183.
229. Pisella PJ, Brignole F, Debbasch C, et al. Flow cytometric analysis of conjunctival epithelium in ocular rosacea and keratoconjunctivitis sicca. *Ophthalmology*. 2000;107(10):1841-1849.
230. Perez VL, Pflugfelder SC, Zhang S, Shojaei A, Haque R. Lifitegrast, a Novel Integrin Antagonist for Treatment of Dry Eye Disease. *Ocul Surf*. 2016;14(2):207-215.
231. Gao J, Morgan G, Tieu D, et al. ICAM-1 expression predisposes ocular tissues to immune-based inflammation in dry eye patients and Sjögrens syndrome-like MRL/lpr mice. *Exp Eye Res*. 2004;78(4):823-835.
232. Ecoiffier T, El Annan J, Rashid S, Schaumberg D, Dana R. Modulation of Integrin α4β1 (VLA-4) in Dry Eye Disease. *Arch Ophthalmol*. 2008;126(12):1695.
233. El Annan J, Chauhan SK, Ecoiffier T, Zhang Q, Saban DR, Dana R. Characterization of effector T cells in dry eye disease. *Invest Ophthalmol Vis Sci*. 2009;50(8):3802-3807.

234. Chauhan SK, Dana R. Role of Th17 cells in the immunopathogenesis of dry eye disease. *Mucosal Immunol*. 2009;2(4):375-376.

235. De Paiva CS, Chotikavanich S, Pangelinan SB, et al. IL-17 disrupts corneal barrier following desiccating stress. *Mucosal Immunol*. 2009;2(3):243-253.

236. Niederkorn JY, Stern ME, Pflugfelder SC, et al. Desiccating Stress Induces T Cell-Mediated Sjögren's Syndrome-Like Lacrimal Keratoconjunctivitis. *J Immunol*. 2006;176(7).

237. Kunert KS, Tisdale AS, Stern ME, Smith JA, Gipson IK. Analysis of topical cyclosporine treatment of patients with dry eye syndrome: effect on conjunctival lymphocytes. *Arch Ophthalmol (Chicago, Ill 1960)*. 2000;118(11):1489-1496.

238. Kymionis GD, Bouzoukis DI, Diakonis VF, Siganos C. Treatment of chronic dry eye: focus on cyclosporine. *Clin Ophthalmol*. 2008;2(4):829-836.

239. Rashid S, Jin Y, Ecoiffier T, Barabino S, Schaumberg DA, Dana MR. Topical Omega-3 and Omega-6 Fatty Acids for Treatment of Dry Eye. *Arch Ophthalmol*. 2008;126(2):219.

240. Goyal S, Chauhan SK, El Annan J, Nallasamy N, Zhang Q, Dana R. Evidence of Corneal Lymphangiogenesis in Dry Eye Disease. *Arch Ophthalmol*. 2010;128(7):819.

241. Goyal S, Chauhan SK, Zhang Q, Dana R. Amelioration of Murine Dry Eye Disease by Topical Antagonist to Chemokine Receptor 2. *Arch Ophthalmol*. 2009;127(7):882.

242. Schaumburg CS, Siemasko KF, De Paiva CS, et al. Ocular Surface APCs Are Necessary for Autoreactive T Cell-Mediated Experimental Autoimmune Lacrimal Keratoconjunctivitis. *J Immunol*. 2011;187(7):3653-3662.

243. Villani E, Galimberti D, Viola F, Mapelli C, Ratiglia R. The Cornea in Sjögren's Syndrome: An In Vivo Confocal Study. *Investig Opthalmology Vis Sci*. 2007;48(5):2017.

244. Lin H, Li W, Dong N, et al. Changes in Corneal Epithelial Layer Inflammatory

Cells in Aqueous Tear–Deficient Dry Eye. *Investig Opthalmology Vis Sci*. 2010;51(1):122.

245. Chauhan SK, El Annan J, Ecoiffier T, et al. Autoimmunity in dry eye is due to resistance of Th17 to Treg suppression. *J Immunol*. 2009;182(3):1247-1252.
246. Amadi-Obi A, Yu C-R, Liu X, et al. TH17 cells contribute to uveitis and scleritis and are expanded by IL-2 and inhibited by IL-27/STAT1. *Nat Med*. 2007;13(6):711-718.
247. Abraham C, Cho J. Interleukin-23/Th17 pathways and inflammatory bowel disease. *Inflamm Bowel Dis*. 2009;15(7):1090-1100.
248. Hirota K, Yoshitomi H, Hashimoto M, et al. Preferential recruitment of CCR6-expressing Th17 cells to inflamed joints via CCL20 in rheumatoid arthritis and its animal model. *J Exp Med*. 2007;204(12):2803-2812.
249. Kebir H, Kreymborg K, Ifergan I, et al. Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. *Nat Med*. 2007;13(10):1173-1175.
250. Chen Y, Chauhan SK, Shao C, Omoto M, Inomata T, Dana R. IFN-γ-Expressing Th17 Cells Are Required for Development of Severe Ocular Surface Autoimmunity. *J Immunol*. June 2017:ji1602144.
251. Subbarayal B, Chauhan SK, Di Zazzo A, Dana R. IL-17 Augments B Cell Activation in Ocular Surface Autoimmunity. *J Immunol*. 2016;197(9):3464-3470.
252. Kodati S, Chauhan SK, Chen Y, et al. CCR7 is critical for the induction and maintenance of Th17 immunity in dry eye disease. *Invest Ophthalmol Vis Sci*. 2014;55(9):5871-5877.
253. Chen Y, Chauhan SK, Soo Lee H, Saban DR, Dana R. Chronic dry eye disease is principally mediated by effector memory Th17 cells. *Mucosal Immunol*. 2014;7(1):38-45.
254. Chen Y, Chauhan SK, Tan X, Dana R. Interleukin-7 and -15 maintain pathogenic memory Th17 cells in autoimmunity. *J Autoimmun*. 2017;77:96-103.

255. Haines CJ, Chen Y, Blumenschein WM, et al. Autoimmune memory T helper 17 cell function and expansion are dependent on interleukin-23. *Cell Rep*. 2013;3(5):1378-1388.

256. Sakaguchi S, Sakaguchi N, Shimizu J, et al. Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol Rev*. 2001;182(1):18-32.

257. Niederkorn JY, Stern ME, Pflugfelder SC, et al. Desiccating stress induces T cell-mediated Sjögren's Syndrome-like lacrimal keratoconjunctivitis. *J Immunol*. 2006;176(7):3950-3957.

258. Siemasko KF, Gao J, Calder VL, et al. In Vitro Expanded CD4+CD25+Foxp3+ Regulatory T Cells Maintain a Normal Phenotype and Suppress Immune-Mediated Ocular Surface Inflammation. *Investig Opthalmology Vis Sci*. 2008;49(12):5434.

259. Sun L, Hurez VJ, Thibodeaux SR, et al. Aged regulatory T cells protect from autoimmune inflammation despite reduced STAT3 activation and decreased constraint of IL-17 producing T cells. *Aging Cell*. 2012;11(3):509-519.

260. Coursey TG, Bian F, Zaheer M, Pflugfelder SC, Volpe EA, de Paiva CS. Age-related spontaneous lacrimal keratoconjunctivitis is accompanied by dysfunctional T regulatory cells. *Mucosal Immunol*. October 2016.

261. Di Zazzo A, Tahvildari M, Subbarayal B, et al. Proangiogenic Function of T Cells in Corneal Transplantation. *Transplantation*. August 2016:1.

262. Tan X, Chen Y, Foulsham W, et al. The immunoregulatory role of corneal epithelium-derived thrombospondin-1 in dry eye disease. *Ocul Surf*. 2018;16(4):470-477.

263. Dohlman TH, Chauhan SK, Kodati S, et al. The CCR6/CCL20 axis mediates Th17 cell migration to the ocular surface in dry eye disease. *Invest Ophthalmol Vis Sci*. 2013;54(6):4081-4091.

264. Chen Y, Chauhan SK, Saban DR, Sadrai Z, Okanobo A, Dana R. Interferon-γ-secreting NK cells promote induction of dry eye disease. *J Leukoc Biol*. 2011;89(6):965-972.

265. Salabarria A-C, Braun G, Heykants M, et al. Local VEGF-A blockade modulates the microenvironment of the corneal graft bed. *Am J Transplant*. March 2019.

266. Amescua G, Collings F, Sidani A, 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):615-625.

267. Inomata T, Mashaghi A, Di Zazzo A, Dana R. Ocular surgical models for immune and angiogenic responses. *J Biol methods*. 2015;2(3):27.

268. Saban DR, Bock F, Chauhan SK, Masli S, Dana R. Thrombospondin-1 derived from APCs regulates their capacity for allosensitization. *J Immunol*. 2010;185(8):4691-4697.

269. Sonoda Y, Streilein JW. Orthotopic corneal transplantation in mice--evidence that the immunogenetic rules of rejection do not apply. *Transplantation*. 1992;54(4):694-704.

270. Blanco-Mezquita JT, Hutcheon AEK, Stepp MA, Zieske JD. αVβ6 Integrin Promotes Corneal Wound Healing. *Investig Opthalmology Vis Sci*. 2011;52(11):8505.

271. Mittal SK, Mashaghi A, Amouzegar A, et al. Mesenchymal Stromal Cells Inhibit Neutrophil Effector Functions in a Murine Model of Ocular Inflammation. *Invest Ophthalmol Vis Sci*. 2018;59(3):1191-1198.

272. Guzmán M, Keitelman I, Sabbione F, Trevani AS, Giordano MN, Galletti JG. Desiccating stress-induced disruption of ocular surface immune tolerance drives dry eye disease. *Clin Exp Immunol*. 2016;184(2):248-256.

273. Dursun D, Wang M, Monroy D, et al. A mouse model of keratoconjunctivitis sicca. *Invest Ophthalmol Vis Sci*. 2002;43(3):632-638.

274. Barabino S, Shen L, Chen L, Rashid S, Rolando M, Dana MR. The Controlled-

Environment Chamber: A New Mouse Model of Dry Eye. *Investig Opthalmology Vis Sci*. 2005;46(8):2766.

275. Yoon K-C, De Paiva CS, Qi H, et al. Expression of Th-1 Chemokines and Chemokine Receptors on the Ocular Surface of C57BL/6 Mice: Effects of Desiccating Stress. *Investig Opthalmology Vis Sci*. 2007;48(6):2561.
276. Stern ME, Pflugfelder SC. What We Have Learned From Animal Models of Dry Eye. *Int Ophthalmol Clin*. 2017;57(2):109-118.
277. Gao J, Morgan G, Tieu DD, Schwalb TA, Ngo M, Stern ME. ICAM-1: its role in the pathophysiology of immune activation in the MRL/LPR mouse. *Adv Exp Med Biol*. 2002;506(Pt B):777-781.
278. Yoon K-C, Park C-S, You I-C, et al. Expression of CXCL9, -10, -11, and CXCR3 in the Tear Film and Ocular Surface of Patients with Dry Eye Syndrome. *Investig Opthalmology Vis Sci*. 2010;51(2):643.
279. Jones DT, Monroy D, Ji Z, Atherton SS, Pflugfelder SC. Sjögren's syndrome: cytokine and Epstein-Barr viral gene expression within the conjunctival epithelium. *Invest Ophthalmol Vis Sci*. 1994;35(9):3493-3504.
280. Gao J, Schwalb TA, Addeo J V, Ghosn CR, Stern ME. The role of apoptosis in the pathogenesis of canine keratoconjunctivitis sicca: the effect of topical Cyclosporin A therapy. *Cornea*. 1998;17(6):654-663.
281. Yeh S, Song XJ, Farley W, Li D-Q, Stern ME, Pflugfelder SC. Apoptosis of ocular surface cells in experimentally induced dry eye. *Invest Ophthalmol Vis Sci*. 2003;44(1):124-129.
282. Brignole F, Pisella PJ, De Saint Jean M, Goldschild M, Goguel A, Baudouin C. Flow cytometric analysis of inflammatory markers in KCS: 6-month treatment with topical cyclosporin A. *Invest Ophthalmol Vis Sci*. 2001;42(1):90-95.
283. Corrales RM, de Paiva CS, Li D-Q, et al. Entrapment of Conjunctival Goblet Cells by Desiccation-Induced Cornification. *Investig Opthalmology Vis Sci*. 2011;52(6):3492.

284. De Paiva CS, Villarreal AL, Corrales RM, et al. Dry Eye–Induced Conjunctival Epithelial Squamous Metaplasia Is Modulated by Interferon-γ. *Investig Opthalmology Vis Sci*. 2007;48(6):2553.

285. Pflugfelder SC, Tseng SC, Yoshino K, Monroy D, Felix C, Reis BL. Correlation of goblet cell density and mucosal epithelial membrane mucin expression with rose bengal staining in patients with ocular irritation. *Ophthalmology*. 1997;104(2):223-235.

286. Pflugfelder SC, De Paiva CS, Moore QL, et al. Aqueous Tear Deficiency Increases Conjunctival Interferon-γ (IFN-γ) Expression and Goblet Cell Loss. *Investig Opthalmology Vis Sci*. 2015;56(12):7545.

287. Lee HS, Amouzegar A, Dana R. Kinetics of Corneal Antigen Presenting Cells in Experimental Dry Eye Disease. *BMJ Open Ophthalmol*. 2017;1(1):e000078.

288. Sheppard JD, Singh R, McClellan AJ, et al. Long-term Supplementation With n-6 and n-3 PUFAs Improves Moderate-to-Severe Keratoconjunctivitis Sicca. *Cornea*. 2013;32(10):1297-1304.

289. Sadrai Z, Stevenson W, Okanobo A, et al. PDE4 inhibition suppresses IL-17-associated immunity in dry eye disease. *Invest Ophthalmol Vis Sci*. 2012;53(7):3584-3591.

290. Pflugfelder SC, Maskin SL, Anderson B, et al. A randomized, double-masked, placebo-controlled, multicenter comparison of loteprednol etabonate ophthalmic suspension, 0.5%, and placebo for treatment of keratoconjunctivitis sicca in patients with delayed tear clearance. *Am J Ophthalmol*. 2004;138(3):444-457.

291. Moore QL, De Paiva CS, Pflugfelder SC. Effects of Dry Eye Therapies on Environmentally Induced Ocular Surface Disease. *Am J Ophthalmol*. 2015;160(1):135-142.e1.

292. Sheppard JD, Donnenfeld ED, Holland EJ, et al. Effect of Loteprednol Etabonate 0.5% on Initiation of Dry Eye Treatment With Topical Cyclosporine 0.05%. *Eye Contact Lens Sci Clin Pract*. 2014;40(5):289-296.

293. Strong B, Farley W, Stern ME, Pflugfelder SC. Topical cyclosporine inhibits conjunctival epithelial apoptosis in experimental murine keratoconjunctivitis sicca. *Cornea*. 2005;24(1):80-85.

294. Kunert KS, Tisdale AS, Gipson IK. Goblet Cell Numbers and Epithelial Proliferation in the Conjunctiva of Patients With Dry Eye Syndrome Treated With Cyclosporine. *Arch Ophthalmol*. 2002;120(3):330.

295. Pflugfelder SC, De Paiva CS, Villarreal AL, Stern ME. Effects of Sequential Artificial Tear and Cyclosporine Emulsion Therapy on Conjunctival Goblet Cell Density and Transforming Growth Factor-β2 Production. *Cornea*. 2008;27(1):64-69.

296. Barabino S, Dana MR. Animal Models of Dry Eye: A Critical Assessment of Opportunities and Limitations. *Investig Opthalmology Vis Sci*. 2004;45(6):1641.

297. Chen Y, Chauhan SK, Lee HS, et al. Effect of desiccating environmental stress versus systemic muscarinic AChR blockade on dry eye immunopathogenesis. *Invest Ophthalmol Vis Sci*. 2013;54(4):2457-2464.

298. Fujii T, Kawashima K. Ca2+ oscillation and c-fos gene expression induced via muscarinic acetylcholine receptor in human T- and B-cell lines. *Naunyn Schmiedebergs Arch Pharmacol*. 2000;362(1):14-21.

299. Nomura J, Hosoi T, Okuma Y, Nomura Y. The presence and functions of muscarinic receptors in human T cells: the involvement in IL-2 and IL-2 receptor system. *Life Sci*. 2003;72(18-19):2121-2126.

300. Razani-Boroujerdi S, Behl M, Hahn FF, Pena-Philippides JC, Hutt J, Sopori ML. Role of muscarinic receptors in the regulation of immune and inflammatory responses. *J Neuroimmunol*. 2008;194(1-2):83-88.

301. Arzt ES, Fernández-Castelo S, Diaz A, Finkielman S, Nahmod VE. The muscarinic agonist pilocarpine inhibits DNA and interferon-gamma synthesis in peripheral blood mononuclear cells. *Int J Immunopharmacol*. 1989;11(3):275-281.

302. Lemp MA. Report of the National Eye Institute/Industry workshop on Clinical Trials in Dry Eyes. *CLAO J*. 1995;21(4):221-232.

303. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*. 2006;8(4):315-317.

304. Mittal SK, Foulsham W, Shukla S, Elbasiony E, Omoto M, Chauhan SK. Mesenchymal Stromal Cells Modulate Corneal Alloimmunity via Secretion of Hepatocyte Growth Factor. *Stem Cells Transl Med*. June 2019:sctm.19-0004.

305. Janeway CA. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol*. 1989;54 Pt 1:1-13.

306. Jain A, Pasare C. Innate Control of Adaptive Immunity: Beyond the Three-Signal Paradigm. *J Immunol*. 2017;198(10):3791-3800.

307. Cekic C, Linden J. Purinergic regulation of the immune system. *Nat Rev Immunol*. 2016;16(3):177-192.

308. Eltzschig HK, Sitkovsky M V., Robson SC. Purinergic Signaling during Inflammation. *N Engl J Med*. 2012;367(24):2322-2333.

309. Junger WG. Immune cell regulation by autocrine purinergic signalling. *Nat Rev Immunol*. 2011;11(3):201-212.

310. Zimmermann H. Extracellular ATP and other nucleotides—ubiquitous triggers of intercellular messenger release. *Purinergic Signal*. 2016;12(1):25-57.

311. Sandilos JK, Chiu Y-H, Chekeni FB, et al. Pannexin 1, an ATP Release Channel, Is Activated by Caspase Cleavage of Its Pore-associated C-terminal Autoinhibitory Region. *J Biol Chem*. 2012;287(14):11303-11311.

312. Ferrari D, Pizzirani C, Adinolfi E, et al. The P2X7 receptor: a key player in IL-1 processing and release. *J Immunol*. 2006;176(7):3877-3883.

313. Yip L, Woehrle T, Corriden R, et al. Autocrine regulation of T-cell activation by ATP release and P2X7 receptors. *FASEB J*. 2009;23(6):1685-1693.

314. Ohta A, Sitkovsky M. Role of G-protein-coupled adenosine receptors in

downregulation of inflammation and protection from tissue damage. *Nature*. 2001;414(6866):916-920.

315. Vergani A, Tezza S, D'Addio F, et al. Long-Term Heart Transplant Survival by Targeting the Ionotropic Purinergic Receptor P2X7. *Circulation*. 2013;127(4):463-475.
316. Liu K, Vergani A, Zhao P, et al. Inhibition of the Purinergic Pathway Prolongs Mouse Lung Allograft Survival. *Am J Respir Cell Mol Biol*. March 2014:140324142513008.
317. Vergani A, Fotino C, D'Addio F, et al. Effect of the purinergic inhibitor oxidized ATP in a model of islet allograft rejection. *Diabetes*. 2013;62(5):1665-1675.
318. Koo TY, Lee J-G, Yan J-J, et al. The P2X7 receptor antagonist, oxidized adenosine triphosphate, ameliorates renal ischemia-reperfusion injury by expansion of regulatory T cells. *Kidney Int*. April 2017.
319. Schenk U, Frascoli M, Proietti M, et al. ATP inhibits the generation and function of regulatory T cells through the activation of purinergic P2X receptors. *Sci Signal*. 2011;4(162):ra12.
320. Jia L, Liu Y, Wang L, Zhu J, Huang Y. Effects of topical sphingosine-1-phosphate 1 receptor agonist on corneal allograft in mice. *Cornea*. 2014;33(4):398-404.
321. Cursiefen C, Maruyama K, Jackson DG, Streilein JW, Kruse FE. Time course of angiogenesis and lymphangiogenesis after brief corneal inflammation. *Cornea*. 2006;25(4):443-447.
322. Li M, Mittal SK, Foulsham W, Amouzegar A, Sahu SK, Chauhan SK. Mast cells contribute to the induction of ocular mucosal alloimmunity. *Am J Transplant*. August 2018.
323. Satitpitakul V, Sun Z, Suri K, et al. Vasoactive Intestinal Peptide Promotes Corneal Allograft Survival. *Am J Pathol*. 2018;188(9):2016-2024.
324. Nakakura EK, Shorthouse RA, Zheng B, McCabe SM, Jardieu PM, Morris RE.

Long-term survival of solid organ allografts by brief anti-lymphocyte function-associated antigen-1 monoclonal antibody monotherapy. *Transplantation*. 1996;62(5):547-552.

325. Murase N, Kim DG, Todo S, Cramer D V, Fung JJ, Starzl TE. Suppression of allograft rejection with FK506. I. Prolonged cardiac and liver survival in rats following short-course therapy. *Transplantation*. 1990;50(2):186-189.
326. Markees TG, Phillips NE, Noelle RJ, et al. Prolonged survival of mouse skin allografts in recipients treated with donor splenocytes and antibody to CD40 ligand. *Transplantation*. 1997;64(2):329-335.
327. Tanriover B, Zhang S, MacConmara M, et al. Induction Therapies in Live Donor Kidney Transplantation on Tacrolimus and Mycophenolate With or Without Steroid Maintenance. *Clin J Am Soc Nephrol*. 2015;10(6):1041-1049.
328. Laftavi MR, Sharma R, Feng L, Said M, Pankewycz O. Induction Therapy in Renal Transplant Recipients: A Review. *Immunol Invest*. 2014;43(8):790-806.
329. Sonoda Y, Sano Y, Ksander B, Streilein JW. Characterization of cell-mediated immune responses elicited by orthotopic corneal allografts in mice. *Invest Ophthalmol Vis Sci*. 1995;36(2):427-434.
330. Inomata T, Hua J, Nakao T, et al. Corneal Tissue from Dry Eye Donors Leads to Enhanced Graft Rejection. *Cornea*. 2018;37(1):95-101.
331. Amouzegar A, Mittal SK, Sahu A, Sahu SK, Chauhan SK. Mesenchymal Stem Cells Modulate Differentiation of Myeloid Progenitor Cells During Inflammation. *Stem Cells*. 2017;35(6):1532-1541.
332. Wang Y, Chen X, Cao W, Shi Y. Plasticity of mesenchymal stem cells in immunomodulation: pathological and therapeutic implications. *Nat Immunol*. 2014;15(11):1009-1016.
333. Lutz MB, Schuler G. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends Immunol*. 2002;23(9):445-449.

334. English K, Barry FP, Mahon BP. Murine mesenchymal stem cells suppress dendritic cell migration, maturation and antigen presentation. *Immunol Lett*. 2008;115(1):50-58.

335. Jacobs SA, Roobrouck VD, Verfaillie CM, Van Gool SW. Immunological characteristics of human mesenchymal stem cells and multipotent adult progenitor cells. *Immunol Cell Biol*. 2013;91(1):32-39.

336. Oh K, Iimuro Y, Takeuchi M, et al. Ameliorating effect of hepatocyte growth factor on inflammatory bowel disease in a murine model. *Am J Physiol Liver Physiol*. 2005;288(4):G729-G735.

337. Futamatsu H, Suzuki J, Mizuno S, et al. Hepatocyte Growth Factor Ameliorates the Progression of Experimental Autoimmune Myocarditis. *Circ Res*. 2005;96(8):823-830.

338. Okunishi K, Dohi M, Nakagome K, et al. A novel role of hepatocyte growth factor as an immune regulator through suppressing dendritic cell function. *J Immunol*. 2005;175(7):4745-4753.

339. Okunishi K, Dohi M, Fujio K, et al. Hepatocyte growth factor significantly suppresses collagen-induced arthritis in mice. *J Immunol*. 2007;179(8):5504-5513.

340. Yamaura K, Ito K, Tsukioka K, et al. Suppression of acute and chronic rejection by hepatocyte growth factor in a murine model of cardiac transplantation: induction of tolerance and prevention of cardiac allograft vasculopathy. *Circulation*. 2004;110(12):1650-1657.

341. Kuroiwa T, Iwasaki T, Imado T, Sekiguchi M, Fujimoto J, Sano H. Hepatocyte growth factor prevents lupus nephritis in a murine lupus model of chronic graft-versus-host disease. *Arthritis Res Ther*. 2006;8(4):R123.

342. Kuroiwa T, Kakishita E, Hamano T, et al. Hepatocyte growth factor ameliorates acute graft-versus-host disease and promotes hematopoietic function. *J Clin Invest*. 2001;107(11):1365-1373.

343. Basu S, Hertsenberg AJ, Funderburgh ML, et al. Human limbal biopsy-derived stromal stem cells prevent corneal scarring. *Sci Transl Med*. 2014;6(266):266ra172.

344. Mundra V, Gerling IC, Mahato RI. Mesenchymal Stem Cell-Based Therapy. *Mol Pharm*. 2013;10(1):77-89.

345. Bagno L, Hatzistergos KE, Balkan W, Hare JM. Mesenchymal Stem Cell-Based Therapy for Cardiovascular Disease: Progress and Challenges. *Mol Ther*. 2018;26(7):1610-1623.

346. English K. Mechanisms of mesenchymal stromal cell immunomodulation. *Immunol Cell Biol*. 2013;91(1):19-26.

347. Fulop T, Larbi A, Dupuis G, et al. Immunosenescence and Inflamm-Aging As Two Sides of the Same Coin: Friends or Foes? *Front Immunol*. 2017;8:1960.

348. Fulop T, Dupuis G, Baehl S, et al. From inflamm-aging to immune-paralysis: a slippery slope during aging for immune-adaptation. *Biogerontology*. 2016;17(1):147-157.

349. Franceschi C, Bonafè M, Valensin S, et al. Inflamm-aging. An evolutionary perspective on immunosenescence. *Ann N Y Acad Sci*. 2000;908:244-254.

350. Rea IM, Gibson DS, McGilligan V, McNerlan SE, Alexander HD, Ross OA. Age and Age-Related Diseases: Role of Inflammation Triggers and Cytokines. *Front Immunol*. 2018;9:586.

351. Lynch HE, Goldberg GL, Chidgey A, Van den Brink MRM, Boyd R, Sempowski GD. Thymic involution and immune reconstitution. *Trends Immunol*. 2009;30(7):366-373.

352. McKay D, Jameson J. Kidney transplantation and the ageing immune system. *Nat Rev Nephrol*. 2012;8(12):700-708.

353. Yager EJ, Ahmed M, Lanzer K, Randall TD, Woodland DL, Blackman MA. Age-associated decline in T cell repertoire diversity leads to holes in the repertoire and impaired immunity to influenza virus. *J Exp Med*. 2008;205(3):711-723.

354. Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol*. 2007;8(9):729-740.

355. Della Bella S, Bierti L, Presicce P, et al. Peripheral blood dendritic cells and monocytes are differently regulated in the elderly. *Clin Immunol*. 2007;122(2):220-228.

356. Agrawal A, Agrawal S, Cao J-N, Su H, Osann K, Gupta S. Altered innate immune functioning of dendritic cells in elderly humans: a role of phosphoinositide 3-kinase-signaling pathway. *J Immunol*. 2007;178(11):6912-6922.

357. Mahbub S, L. Brubaker A, J. Kovacs E. Aging of the Innate Immune System: An Update. *Curr Immunol Rev*. 2011;7(1):104-115.

358. Maria, Ni E, Roda P, et al. Age-associated changes in CD8+ and CD16+ cell reactivity: clonal analysis. *Clin Exp Immunol*. 2008;81(3):479-484.

359. Mocchegiani E, Malavolta M. NK and NKT cell functions in immunosenescence. *Aging Cell*. 2004;3(4):177-184.

360. Gipson IK, MM R, PR. L, LW H, AE. M. Age-related changes and diseases of the ocular surface and cornea. 2013;54(14).

361. Wei A, Hong J, Sun X, Xu J. Evaluation of Age-related Changes in Human Palpebral Conjunctiva and Meibomian Glands by In Vivo Confocal Microscopy. *Cornea*. 2011;30(9):1007-1012.

362. Furukawa RE, Polse KA. Changes in tear flow accompanying aging. *Am J Optom Physiol Opt*. 1978;55(2):69-74.

363. Nasu M, Matsubara O, Yamamoto H. Post-mortem prevalence of lymphocytic infiltration of the lacrymal gland: A comparative study in autoimmune and non-autoimmune diseases. *J Pathol*. 1984;143(1):11-15.

364. Obata H, Yamamoto S, Horiuchi H, Machinami R. Histopathologic study of human lacrimal gland. Statistical analysis with special reference to aging. *Ophthalmology*. 1995;102(4):678-686.

365. Stepp MA, Pal-Ghosh S, Tadvalkar G, Williams A, Pflugfelder SC, de Paiva CS. Reduced intraepithelial corneal nerve density and sensitivity accompany desiccating stress and aging in C57BL/6 mice. *Exp Eye Res*. 2018;169:91-98.

366. Knop E, Knop N, Millar T, Obata H, Sullivan DA. The International Workshop on Meibomian Gland Dysfunction: Report of the Subcommittee on Anatomy, Physiology, and Pathophysiology of the Meibomian Gland. *Investig Opthalmology Vis Sci*. 2011;52(4):1938.

367. Micera A, Di Zazzo A, Esposito G, et al. Age-Related Changes to Human Tear Composition. *Investig Opthalmology Vis Sci*. 2018;59(5):2024.

368. McClellan AJ, Volpe EA, Zhang X, et al. Ocular Surface Disease and Dacryoadenitis in Aging C57BL/6 Mice. *Am J Pathol*. 2014;184(3):631-643.

369. Weng N-P. Aging of the immune system: how much can the adaptive immune system adapt? *Immunity*. 2006;24(5):495-499.

370. Devarajan P, Chen Z. Autoimmune effector memory T cells: the bad and the good. *Immunol Res*. 2013;57(1-3):12-22.

371. McGeachy MJ. Th17 memory cells: live long and proliferate. *J Leukoc Biol*. 2013;94(5):921-926.

372. Burkett PR, Horste GM zu, Kuchroo VK. Pouring fuel on the fire: Th17 cells, the environment, and autoimmunity. *J Clin Invest*. 2015;125(6):2211-2219.

373. Kryczek I, Zhao E, Liu Y, et al. Human TH17 Cells Are Long-Lived Effector Memory Cells. *Sci Transl Med*. 2011;3(104).

374. Leipe J, Grunke M, Dechant C, et al. Role of Th17 cells in human autoimmune arthritis. *Arthritis Rheum*. 2010;62(10):2876-2885.

375. Fitch E, Harper E, Skorcheva I, Kurtz SE, Blauvelt A. Pathophysiology of psoriasis: recent advances on IL-23 and Th17 cytokines. *Curr Rheumatol Rep*. 2007;9(6):461-467.

376. Clark RA. Resident memory T cells in human health and disease. *Sci Transl Med*. 2015;7(269):269rv1.

377. Liu R, Gao C, Chen H, Li Y, Jin Y, Qi H. Analysis of Th17-associated cytokines and clinical correlations in patients with dry eye disease. *PLoS One*. 2017;12(4):e0173301.

378. Pflugfelder SC, Stern M, Zhang S, Shojaei A. LFA-1/ICAM-1 Interaction as a Therapeutic Target in Dry Eye Disease. *J Ocul Pharmacol Ther*. 2017;33(1):5-12.

379. Barbosa FL, Xiao Y, Bian F, et al. Goblet Cells Contribute to Ocular Surface Immune Tolerance-Implications for Dry Eye Disease. *Int J Mol Sci*. 2017;18(5).

380. Berard M, Brandt K, Bulfone-Paus S, Tough DF. IL-15 promotes the survival of naive and memory phenotype CD8+ T cells. *J Immunol*. 2003;170(10):5018-5026.

381. Stonier SW, Ma LJ, Castillo EF, Schluns KS. Dendritic cells drive memory CD8 T-cell homeostasis via IL-15 transpresentation. *Blood*. 2008;112(12):4546-4554.

382. Saban DR, Chauhan SK, Zhang X, El Annan J, Jin Y, Dana R. “Chimeric” grafts assembled from multiple allodisparate donors enjoy enhanced transplant survival. *Am J Transplant*. 2009;9(3):473-482.

383. Cursiefen C, Chen L, Dana MR, Streilein JW. Corneal lymphangiogenesis: evidence, mechanisms, and implications for corneal transplant immunology. *Cornea*. 2003;22(3):273-281.

384. Hamrah P, Haskova Z, Taylor AW, Zhang Q, Ksander BR, Dana MR. Local treatment with alpha-melanocyte stimulating hormone reduces corneal allorejection. *Transplantation*. 2009;88(2):180-187.

385. Costello R, Duffaud F. The pleiotropic effects of interleukin 7 and their pathologic and therapeutic implications. *Eur J Med*. 1992;1(2):119-121.

386. Schenk U, Westendorf AM, Radaelli E, et al. Purinergic Control of T Cell Activation by ATP Released Through Pannexin-1 Hemichannels. *Sci Signal*. 2008;1(39):ra6-ra6.

387. Zhao R, Liang D, Sun D. Blockade of Extracellular ATP Effect by Oxidized ATP

Effectively Mitigated Induced Mouse Experimental Autoimmune Uveitis (EAU). Rosenbaum JT, ed. *PLoS One*. 2016;11(5):e0155953.

388. Ardissone V, Radaelli E, Zaratin P, et al. Pharmacologic P2X purinergic receptor antagonism in the treatment of collagen-induced arthritis. *Arthritis Rheum*. 2011;63(11):3323-3332.
389. Lang PA, Merkler D, Funkner P, et al. Oxidized ATP inhibits T-cell-mediated autoimmunity. *Eur J Immunol*. 2010;40(9):2401-2408.
390. Huq S, Liu Y, Benichou G, Dana MR. Relevance of the direct pathway of sensitization in corneal transplantation is dictated by the graft bed micronenvironment. *J Immunol*. 2004;173:4464-4469.
391. Di Virgilio F. Novel data point to a broader mechanism of action of oxidized ATP: the P2X7 receptor is not the only target. *Br J Pharmacol*. 2003;140(3):441-443.
392. Bussolino F, Di Renzo MF, Ziche M, et al. Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. *J Cell Biol*. 1992;119(3):629-641.
393. Xin X, Yang S, Ingle G, et al. Hepatocyte growth factor enhances vascular endothelial growth factor-induced angiogenesis in vitro and in vivo. *Am J Pathol*. 2001;158(3):1111-1120.
394. Sandquist I, Kolls J. Update on regulation and effector functions of Th17 cells. *F1000Research*. 2018;7:205.
395. Dardalhon V, Korn T, Kuchroo VK, Anderson AC. Role of Th1 and Th17 cells in organ-specific autoimmunity. *J Autoimmun*. 2008;31(3):252-256.
396. Louveau A, Harris TH, Kipnis J. Revisiting the Mechanisms of CNS Immune Privilege. *Trends Immunol*. 2015;36(10):569-577.
397. Zhou R, Caspi RR. Ocular immune privilege. *F1000 Biol Rep*. 2010;2.
398. Waldmann TA, Tagaya Y. The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host

response to intracellular pathogens. *Annu Rev Immunol*. 1999;17(1):19-49.