Clinical and experimental studies on the cellular mediators of corneal allograft rejection

by

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STATEMENT OF ORIGINALITY

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

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ABSTRACT

Despite significant advances in our knowledge of the cellular and molecular elements of transplant immunology the 10 year survival probability for all human corneal grafts is 0.73. In some "high-risk" recipients it is as low as 0.37. To date almost all our knowledge about the cellular events during acute corneal graft rejection comes from animal models.

In mice, the presence of pre-existing host corneal vascularisation confers "high-risk" status on a graft and has been shown to accelerate rejection. In the first part of this thesis the effect on survival of grafting to an inflamed *conjunctival* bed was investigated. Using a mouse model of allergic conjunctivitis significantly reduced survival was seen in graft recipients with perioperative conjunctival inflammation. This appeared to be due to the local effects of conjunctivitis rather than systemic effects of allergy/ atopy.

Subsequent experiments investigated the effect of perioperative allergic conjunctivitis on the cellular components of both early (surgical trauma-induced, alloantigen-independent) and late (alloantigen-dependent; rejection) post-keratoplasty anterior segment inflammation and demonstrated significant effects on both. Grafts recipients with allergic conjunctivitis had significantly greater early post-operative corneal inflammation and associated corneal and conjunctival lymphangiogenesis. Analysis of graft infiltrating cells during rejection in mice confirmed that large numbers of $CD4^+$ cells, $CD8^+$ cells and macrophages were recruited. Flow cytometric analysis of human aqueous during acute endothelial rejection demonstrated for the first time the presence of $CD4^+$ cells, $CD8^+$ cells and a surprisingly high proportion of macrophages therein. In mouse recipients with allergic conjunctivitis eosinophils were found in both the graft itself and the ciliary body during rejection although the role of these cells during rejection is uncertain.

Chemokine analysis during both murine and human corneal graft rejection demonstrated increased expression of the chemokine IP-10 (CXCL-10) suggesting a potentially important role for this protein in the rejection process.

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LIST OF ABBREVIATIONS

ACAID	Anterior chamber-associated immune deviation
Ag	Antigen
AKC	Atopic keratoconjunctivitis
APC	Antigen-presenting cell
ARG	Arginase
BAB	Blood aqueous barrier
BSA	Bovine serum albumin
CALT	Conjunctiva-associated lymphoid tissue
CBA	Cytometric bead array
CCL	Chemokine of the "CC" family
CCR	Receptor for chemokine of the "C" family
CD	Cluster of differentiation
CTL	Cytotoxic T cell response
CX3CR	Receptor for chemokine of the "CX3C" family
CXCL	Chemokine of the "CXC" family
CXCR	Receptor for chemokine of the "CXC" family
DAB	Diaminobenzidine
DC	Dendritic cell
DTH	Delayed-type hypersensitivity
EDTA	Ethylene-diamine-tetracetic acid
ELISA	Enzyme-linked immunosorbent assay
FasL	Fas ligand
Fc	Fragment of crystallisation
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GADPH	Glycaraldehyde-3-phosphate dehydrogenase
ICAM	Intercellular adhesion molecule
ICOS	Inducible costimulatory molecule
IFN-γ	Interferon gamma

Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
IP-10	10 kilodalton interferon-gamma-induced protein
KC	Keratinocyte-derived chemokine
KO	Knockout
LFA	Lymphocyte function-associated antigen
LYVE-1	Lymphatic vessel endothelial hyaluronan receptor
mAb	monoclonal antibody
MBP	Major basic protein
MCP-1	Monocyte-chemotactic protein-1
mH	Minor histocompatibility antigen
MHC	Major histocompatibility complex
MIG	Monokine induced by interferon gamma
MIP-1α	Macrophage inflammatory protein-1 alpha
MIP-1β	Macrophage inflammatory protein-1 beta
MOPS	3-(N-morpholino)propanesulphonic acid
MST	Median survival time
NB	Northern blot
NDS	Normal donkey serum
NK	Natural killer cell
NOS	Nitric oxide synthetase
OCT	Optimal cutting temperature compound
PAC	Perennial allergic conjunctivitis
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PK	Penetrating keratoplasty
PRR	Pattern recognition receptor
RANTES	Regulated upon activation, normal t cell expressed and secreted

RNA	Ribonucleic acid
RPA	Ribonuclease protection assay
RPM	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SAC	Seasonal allergic conjunctivitis
SRW	Short ragweed pollen
SSC	Side scatter
Tc	Cytotoxic T cell
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th	Helper T cell
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
Treg	Regulatory T cell
VEGF	Vascular endothelial growth factor
VEGFR	Receptor for vascular endothelial growth factor
VKC	Vernal keratoconjunctivitis
WT	Wild-type

1. CHAPTER 1: General Introduction

1.1. CORNEAL ANATOMY AND PHYSIOLOGY

1.1.1. Anatomy

The cornea is a transparent dome of tissue which forms the anterior one sixth of the eyeball. It delimits the anterior chamber anteriorly. Its diameter measures approximately 11.7 mm vertically and 12.6 mm horizontally. It is thicker at the periphery (650^{+}µm) than the centre (500-600 µm)(Klyce S.D. and Beuerman R.D., 2009). The cornea has five layers but only three of these are cellular. These layers are the epithelium, the stroma and the endothelium. The other layers, known as Bowman's membrane and Descemet's membrane, constitute the basement membranes of the epithelium and endothelium respectively. The epithelium is stratified squamous and non-keratinised at the surface. The surface epithelial cells are flattened and covered with glycocalyx and mucins which increase the "wetability" of the cornea and contribute to the unusually smooth surface which is important in allowing efficient transmission of light.
The stroma is relatively acellular and represents approximately 90% of the corneal thickness. It consists of approximately 250 lamellae of collagen fibres. The endothelium is a monolayer of flattened cells on the posterior aspect of the cornea. The normal cornea is avascular and contains no lymphatics.

1.1.2. Corneal function

The function of the cornea may be broadly divided into three; one structural and two optical. Firstly, it forms part of the eyeball and so protects the intraocular contents from the external environment. Secondly its unique property of transparency allows transmission of light. Finally it is the most powerful refractive medium in the eye and so helps to focus the transmitted light/ image at the retina. The refractive power of the cornea is a function of its curvature and refractive index. How it allows transmission of light is less clear. Its avascular nature and smooth surface contribute but the most important factor is thought to be the

regular spacing of the collagen lamellae in the stoma which is maintained by keeping stroma at optimal hydration.

1.1.3. Endothelium

The endothelial layer is the layer of greatest importance to corneal transplant immunologists for 2 reasons. Firstly, unlike the cells of the stromal and epithelial layers, endothelial cells have no regenerative capacity. Cells lost, whether due to immune-mediated damage or any other cause, are not replaced. Adjacent cells may spread to fill any "gaps". Secondly, the endothelial cells play an essential role in maintaining corneal clarity by constantly pumping fluid from the stroma and so maintaining the stroma at optimal hydration to allow transmission of light.

The endothelial cell count falls normally with age but the rate of attrition may be accelerated by intraocular surgery, trauma and various inflammatory and non-inflammatory diseases. During childhood the density of endothelial cells is 3000-4000/mm^2 and decreases steadily by 0.6% per year after age 18(Bourne and McLaren, 2004). Following corneal transplantation the endothelial cell count declines at an even faster rate particularly in the early post-operative years. This rapid decline in endothelial cell density occurs even in the absence of rejection and is thought to be due to the combined effects of corneal storage media and surgical trauma(Armitage et al., 2003). Hence, even in the absence of clinical episodes of immune rejection, the cell count may fall below the density required to maintain graft clarity. The minimum density of endothelial cells required to maintain stromal clarity falls within the range 400-700 cells per mm^2. The occurrence of immune-mediated endothelial rejection accelerates the rate of endothelial cell loss even further. Unlike the cells in the epithelial layer, endothelial cells are post-mitotic. Immune rejection of the endothelial layer is therefore a great threat to graft clarity as cells lost to immune-mediated damage are not replaced. Endothelial rejection, left untreated, usually proceeds to

complete destruction of the endothelial layer with resultant loss of graft clarity. Even if treated, a certain proportion of endothelial cells are lost. Therefore single or recurrent episodes of endothelial rejection, even if treated promptly, may lead to graft failure.

1.2. PENETRATING KERATOPLASTY

1.2.1. Penetrating Keratoplasty: Epidemiology

The first human penetrating keratoplasty, performed in 1905 by Eduard Zirm, was for a corneal chemical injury(Zirm, 1989). Since then the procedure has evolved and has been used for a wide variety of corneal conditions.
The cornea is now the most commonly transplanted tissue worldwide. In the United States over 40,000 are performed annually(Darlington et al., 2006,Ghosheh et al., 2007) while in the UK the number of corneal transplants each year exceeds 2000 (UK Transplant, 2008).

1.2.2. Penetrating Keratoplasty: Indications

The vast majority of corneal grafts are undertaken to improve the optical function of the cornea although the technique is occasionally used to improve or maintain the tectonic function. There is some geographical and temporal variation in the indications for PK. Early iris-clipped lenses lead with time to endothelial cell loss and corneal decompensation. Because these lenses were widely used for cataract surgery in North America, pseudophakic bullous keratopathy has been the commonest indication for PK there for the last 20 years and although advances in lens design, surgical equipment and viscoelastic have lead to a decrease in post-cataract surgery corneal decompensation(Ghosheh et al., 2007), it remains a considerable problem(Cosar et al., 2002). The commonest indication for PK outside the United States is keratoconus(Legeais et al.,

2001,Al-Yousuf et al., 2004). Other main indications include corneal dystrophies, traumatic corneal scarring, and regrafting for failed transplants.

1.2.3. Penetrating Keratoplasty: Survival

The 5-year survival rate for corneal grafts for keratoconus is 90%(Coster and Williams, 2005). Survival rates for renal transplants are similar(Loucaidou et al., 2003) but, unlike solid-organ transplants, this figure is achieved for corneal grafts without systemic immunosuppression which may cause serious side-effects. Not all corneal transplant recipients can expect such a good outcome. Large cohort outcome studies have identified a number of factors which, if present in the host, may have a detrimental effect on graft survival(Williams et al., 2006). The primary diagnosis is important. As mentioned, those patients with keratoconus may expect a 5-year survival of 90%. Those patients with pseudophakic bullous keratopathy, however, do less well with a 5-year survival rate of only 60%. The reasons for this difference are not entirely clear. Other risk factors for subsequent graft rejection include a previous ipsilateral failed graft and vascularisation of the host cornea, the latter being probably the most important and the factor most likely to lead to the need for systemic immunosuppression after corneal transplantation.

Another factor which may shorten graft survival, and one which may be related to corneal vascularisation, is ipsilateral ocular inflammation. Data from the Australian Corneal Graft Registry suggest that the timing of this inflammation is important, inflammation at the time of transplantation being the most dangerous(Coster and Williams, 2005). Patients who have risk factors for graft rejection constitute the “high-risk” group for transplantation. The more risk factors and/or the greater the severity of these factors the greater the risk. Many “high-risk” patients require systemic immunosuppression to prevent or delay early rejection. The most commonly used drugs are cyclosporine, tacrolimus, sirolimus and mycophenylate mofetil but these have serious adverse effects and it is

difficult to justify their use in patients who have satisfactory vision in one eye.

1.3. CLINICAL FEATURES OF CORNEAL GRAFT REJECTION

1.3.1. Epithelial Rejection

The cornea has five layers but only three of these are cellular and relevant as targets in the immune response. These layers are the epithelium, the stroma and the endothelium. Unlike rejection of renal transplants, corneal graft rejection can be diagnosed clinically without the need for tissue biopsy. During a rejection episode patients may complain of pain, photophobia or blurred vision. The clinical signs depend on the layer of the cornea in which rejection is occurring. Clinically distinct rejection of individual layers does occur in humans. These have been described in rabbits also by Khoudadoust(Khodadoust and Silverstein, 1969).

In epithelial rejection an elevated curvilinear white/ opaque line is seen in the epithelium. The visual implications of isolated epithelial rejection are not serious as epithelium quickly and constantly regenerates from the limbus and replaces the rejected cells. However epithelial rejection is taken seriously as it proves that the recipient has been sensitised and has the capacity to reject the deeper corneal layers. In practice epithelial rejection is seldom observed in the clinic. There are several explanations for this. Firstly, because patients with isolated epithelial rejection are usually asymptomatic, it is possible that it is underdiagnosed. Secondly, there is a limited window of opportunity for epithelial rejection to occur before the donor epithelium is replaced by host epithelium (corneal epithelium is replaced horizontally from stem cells at the limbus unlike skin epithelium which grow vertically from the basal layer.) In mice the donor epithelium is replaced by recipient by 15 days(Hori and Streilein, 2001). How

long this process takes in humans is uncertain. There is evidence that human donor epithelium may be completely replaced by 3 months post-transplantation(Lagali et al., 2009) but that some donor epithelial cells may persist beyond 1 year(Egarth et al., 2005).

1.3.2. Stromal Rejection

The clinical symptoms and signs of stromal rejection have two patterns. It may manifest as subepithelial opacities which were described first by Krachmer(Krachmer and Alldredge, 1978). These infiltrates have a similar appearance to those seen in viral keratitis but are seen only in the donor cornea and not in the host. This type of stromal rejection is often asymptomatic but like epithelial rejection it may herald the onset of a more severe and visually significant process. Rejection of the deeper stroma results in graft opacification and decreased visual acuity. In practice, deep stromal rejection and endothelial rejection often occur together. There is experimental evidence of repopulation of the stroma in irradiated mice by bone marrow-derived cells(Chinnery et al., 2008). The degree to which and the timing of stromal cell turnover in human cornea remains uncertain but, as in the epithelium, there may be a limited "window of opportunity" for rejection to occur before repopulation by host-derived cells. Human donor stromal and endothelial cells last considerably longer than epithelial cells. In one study donor stromal and endothelial cells were found in all explanted grafts examined up to a period of 32 years post-transplantation(Lagali et al., 2009).

1.3.3. Endothelial rejection

Patients with isolated endothelial rejection may present with pain, photophobia, a red eye and decreased visual acuity. On examination there are visible cells or cell aggregates in the anterior chamber with accompanying ciliary injection.

There may be corneal stromal oedema the severity and extent of which depends on the longevity and severity of the attack and the pattern of endothelial cell loss. Two patterns of endothelial signs may be seen. A line of leukocytes may be seen “marching” across the endothelium leaving dead endothelium with overlying oedematous stroma in its wake. This line often spreads out like a wave from an area of deep vascularisation to the graft host junction. Alternatively a more diffuse corneal oedema may be seen with diffuse keratitic precipitates of variable density. Transplants in which endothelial rejection is untreated or not reversed by treatment become oedematous on account of endothelial cell depopulation, with irreversible loss of transparency. If commenced at an early enough time before functionally significant loss of endothelial cells, local steroid treatment reverses the endothelial rejection episode in most cases. Steroid-resistant rejection is uncommon.

1.4. INNATE IMMUNITY

1.4.1. Barrier function

The body is constantly under threat of infection/ infestation by a wide range of microorganisms (worms, protozoa, fungi, bacteria and viruses). A wide range of innate immune mechanisms have developed to protect against microbial infection. These innate immune mechanisms are immediately available and do not improve with repeated exposure to the microbe.

Microbes are kept outside the body by the barrier function of the skin and mucosa. Additional mechanisms such as cilia and lavage of fluids (eg tears) enhance the protection at the surface of the body. In the eye, constituents of the tear film such as lysozyme and IgA provide additional innate protection against microbes.

1.4.2. Cellular mediators

Microbes which manage to invade the body encounter various types of leukocyte. Macrophages and neutrophils can engulf and destroy (phagocytose) microbes. The microbe is recognised by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) on the cell surface of phagocytic cells. These receptors have a strong affinity for surface molecular structures known as pathogen-associated molecular patterns (PAMPs) which are shared by many infectious agents and low affinity for the molecular patterns on mammalian cell surface.

The term macrophage was coined by Metchnikoff over one hundred years ago(Kaufmann, 2008) and describes the cell in terms of its most superficial structure and function. In the era of molecular identification of cells many different cell surface markers can be used to identify macrophages depending on their state of maturity or activation. These include CD11b, F4/80, MoMa (in mice), CD14, CD68 and CD163. Macrophages have been found to carry out many and varied functions as part of both the innate and acquired immune response including phagocytosis, chemokine production and antigen presentation. Their phenotypic and functional heterogeneity is now well recognised.(Gordon and Taylor, 2005) It is now thought that macrophages and dendritic cells originate from a common myeloid precursor(Auffray et al., 2009). These are released into the blood stream as immature versions known as monocytes and even at this stage phenotypically-recognisable sub-populations are seen which ultimately give rise to either resident tissue macrophages or DCs or to inflammation-elicited macrophages. Further phenotypic heterogeneity arises from microenvironmental stimuli depending on the tissue and its cytokine and chemokine mileu.

Extracellular killing of infectious agents is mediated by Natural Killer (NK) cells and eosinophils. Natural killer cells bind non-specifically to and induce apoptosis

in virus-infected cells. Eosinophils contain cytoplasmic granules with toxic proteins which are released to fight large parasites such as helminths.

1.4.3. Humoral mediators

The complement system is an important cascade of pro-inflammatory protein production which may be activated by the presence of microorganisms. Activated components of the complement system may kill microrganisms directly by attacking the cell membrane or indirectly by enhancing neutrophil chemotaxis and phagocytosis.

1.5. ACQUIRED IMMUNITY

1.5.1. Cellular mediators

Acquired immunity is characterised by specificity for the antigen in question and by enhancement of the immune response on repeated exposure to the antigen. This response in mediated by lymphocytes. Lymphoid tissue contains millions of lymphocytes each of which has a different recognition site for antigen. On recognition of its complementary antigen, a lymphocyte can undergo clonal expansion to produce large numbers of cells with similar specificity for the antigen. In this way the body has a very large number of specific immune responses at its disposal. Important lymphocyte subsets include:
Helper T cells (Th; CD4+ cells): these cells secrete cytokines which activate other cells such as B lymphocytes and cytotoxic T lymphocytes.
Cytotoxic T cells (Tc; CD8+ cells): these destroy cells with intracellular infection (usually viral)
B cells: these cells become plasma cells and produce antibody

1.5.2. Humoral mediators

The humoral mediators of specific immunity, known as antibodies, are produced by plasma cells which are derived from B-lymphocytes following exposure to a specific antigen. Antibodies are generally involved in fighting extracellular infection. Once the antibody binds to the antigen for which it has specificity it may activate the “classical” complement cascade or facilitate phagocytosis.

1.5.3. Major Histocompatibility Complex

Although its physiological function was not discovered until much later, the importance of the major histocopatibility complex (MHC) was recognised early by the pioneers of transplant immunology. In 1937 Peter Gorer described a strain-specific antigen in inbred mice which profoundly affected the survival of allogeneic tumour transplants(Klein, 1986). This discovery was one of the most important steps in the development of human solid organ transplantation. Ironically, the field of human corneal transplantation was already well established by the time Gorer discovered MHC antigens. Even today the benefit of MHC matching remains a matter of some controversy in corneal transplantation.
The molecules within the MHC were originally defined by their ability to promote vigorous rejection of grafts exchanged between different members of a species. It became clear that a small number of gene products had a disproportionately large influence on allograft survival. Known in humans as Human Leukocyte Antigens and in mice as H-2, these cell surface proteins are involved in binding and presentation of protein degradation products to T cell antigen receptors.

For almost 40 years MHC products were known only for their ability to induce graft rejection. Their physiological role, which is to act as cell surface markers which enable infected cells to signal cytotoxic and helper T cells, was not discovered until 1974(Zinkernagel and Doherty, 1974a,Zinkernagel and Doherty,

1974b). These proteins are encoded by the Major Histocompatibility Complex on chromosome six in humans and chromosome 17 in mice. These MHC genes are the most polymorphic in the human genome. More than 1300 alleles are now known to be present at 12 expressed class I and II loci.
Both MHC class I and class II consist of a trans-membrane glycoprotein folded in such a way as to form a "groove". This groove invariably contains a peptide, 8-9 amino acids long in the case of class I and 13 amino acids long in class II. The peptide is normally a self-derived protein degradation product but if the cell is infected or has phagocytosed foreign tissue, the MHC molecules may bear "foreign" peptides.

MHC class I is found on all nucleated cells and plays an important role in the presentation of antigen to CD8 T cells. MHC class II is found on antigen-presenting-cells and is important in the presentation of antigen to CD4 T cells. The MHC-peptide complex interacts with the T cell receptor (TCR), a complex cell surface receptor. CD3 forms an important and invariant part of the TCR.

As well as TCR-MHC-peptide interaction it has become clear that a second signal is required for activation of T cells. Numerous T cell accessory molecules and their ligands have been discovered. Some of these accessory molecules are primarily co-stimulatory in nature while others are primarily adhesive. Co-stimulatory interactions complement or amplify intracellular signalling by the TCR. Adhesive interactions increase the duration of cell-cell contact. The common function of all accessory molecules is to decrease the number/ strength of TCR-MHC-peptide complexes necessary for T cell activation.

Co-stimulatory Molecules (Found on T cells)	Ligands (Found on Antigen-presenting cells)
CD28 (positive co-stimulation)	B7.1 , B7.2 (CD80 , CD86)
ICOS (positive co-stimulation)	ICOSL
CTLA4 (negative co-stimulation)	B7.1 , B7.2 (CD80 , CD86)
PD-1 (negative co-stimulation)	PD-L1, PD-L2

Table 1.1 T cell co-stimulatory molecules

T Cell Molecules	Ligands on APC
CD2	LFA-3 (CD58)
LFA-1	ICAM-1

Table 1.2 Molecules enhancing T cell APC adhesion

1.5.4. T cell tolerance

T cells are not activated by cells bearing self-antigen because in the thymus, during T cell maturation, T cells with strong affinity for self-antigen are deleted (intrathymic clonal deletion). In other words, self-reactive cells undergo a negative selection process in the thymus. The tolerance induced by this process is known as central tolerance and is distinct from peripheral tolerance which occurs when anergy is induced in extrathymic T cells by peripheral antigens. Once the TCR-MHC-peptide complex has come together in peripheral lymphoid tissue, 4 distinct functional outcomes may be elicited.

1. Productive T cell activation
2. No response (Ignorance)

3. Activation-induced cell death (Peripheral deletion)
4. Induction of unresponsiveness to subsequent antigen (Anergy)

The later 3 outcomes lead to peripheral tolerance of the antigen. Which of these outcomes actually occurs depends, largely, on the affinity of the clonotypic TCR for the MHC-peptide in question. Other important factors which may influence the outcome include:

- The state of the T cell (naïve, memory, unresponsive)
- Soluble factors (cytokines, chemokines)
- The interactions between accessory molecules
- The interaction with other cells such as regulatory T cells (Treg; CD4+CD25+ cells)

1.6. IMMUNOBIOLOGY OF CORNEAL ALLOGRAFT REJECTION

1.6.1. Genes v Environment

Transplanted tissue between genetically identical individuals does not undergo immunological rejection. As mentioned, gene products known as histocompatibility antigens can provoke an immune response in genetically non-identical individuals. Therefore, the likelihood of rejection is, to a degree, genetically pre-determined. For most organs, the greater the degree of genetic mismatch the greater the risk of rejection. However, as is the case for most pathological processes, rejection is not entirely dependent on genetics.
Inbred animals provide an excellent way of studying the role of genetic mismatch whilst controlling for other variables. Corneal grafts between mice with major and multiple minor histocompatiblity antigen mismatches are rejected. However, even when the graft is performed using a standardised surgical procedure by the

same surgeon between several pairs of animals using the same donor recipient inbred strain combination, the grafts are not always rejected at the same time. Furthermore, some grafts are not rejected at all during the study period which is typically two to three months(Figure1.1)(Osawa and Streilein, 2005).

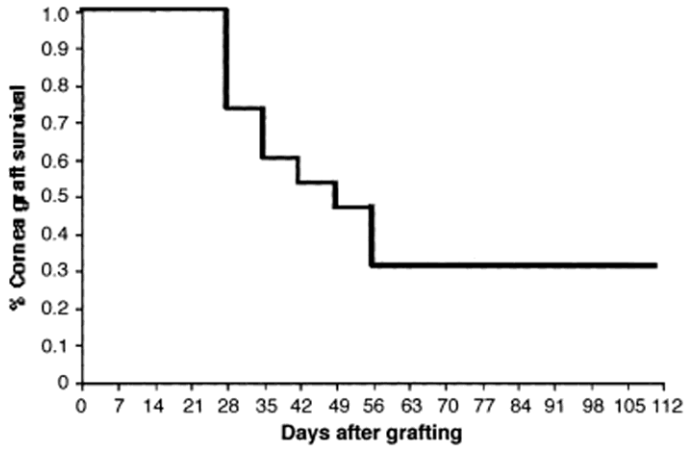


Figure 1.1 Cumulative survival of major and multiple minor H -disparate corneal allografts

Balb/c mice received corneas from C57BL/6 donors. Grafts were rejected at different timepoints with several grafts surviving for 112 days. (Adapted from Osawa H et al Cornea 2005;24;312-318)

These basic data demonstrate that factors other than histo(in)compatablility must influence the process of immune rejection of transplanted tissue. These factors may be especially important in corneal transplantation where, strangely, major histocompatibility matching appears to be of limited value.

Most basic science research in corneal transplantation has focused on the following five questions:

1. What are the cellular and molecular mechanisms involved in the process of immune rejection of corneal tissue?
2. What is the role of MHC/mH matching in corneal transplantation?

3. What are the environmental factors (local & systemic) which influence the survival of corneal grafts?
4. How do these environmental factors affect the cellular/ molecular mechanism(s) of rejection?
5. Can environmental factors (macro environment) and/or molecular processes (micro environment) be modified to improve graft survival?

The latter includes testing the effects of pharmacological interventions.

1.6.2. Surgical trauma and the innate response

Transplant rejection is a classic example of an acquired immune response. However the primary response to all organ grafts is via cellular mediators of innate immunity. Despite advances in surgical technique and suture materials, all organ transplants involve a degree of surgically-induced tissue trauma. In vascularised organs the trauma is due partly to hypoxia/perfusion injury and partly to mechanical trauma. Hypoxia/perfusion injury is less important in the avascular cornea but the net effect of corneal transplantation is the creation of a circumferential full-thickness wound in the cornea between the donor and the host. This induces a wound healing response which is characterised in the first instance by centripetal infiltration of the host cornea by innate immune cells such as neutrophils and macrophages to the tissues adjacent to the wound (Park and Barbul, 2004). This inflammation is alloantigen-independent and occurs early after transplantation. Later, following sensitisation, a rejection episode characterised by inflammation confined to the graft tissue may occur. (Figure 1.2)

There is increasing interest in co-operation between the innate and acquired arms of the immune response and how each may be influenced by the other. The “danger” model proposed by Matzinger proposes that alarm signals activated by innate inflammation (tissue trauma, infection) enhance immunogenicity by activating antigen-presenting cells (APCs)(Matzinger, 2002). As such, the

danger model predicts a role of innate immune cells in the afferent limb of the immune response arc.

Ways in which innate immunity may facilitate a subsequent acquired response include:

- Activation of resident macrophages/ DCs by Toll-like receptor (TLR) or other mechanism
- Cytokine and chemokine release by innately activated macrophages/ DCs
- Recruitment and maturation of cells with antigen presenting capacity
- Recruitment of innate inflammatory cells which express VEGF and drive lymphangiogenesis.

The common clinical observation that a period of alloantigen-independent corneal inflammation such as that caused by a loose corneal suture may trigger an episode of corneal graft rejection in a healthy graft up to 2 or 3 years after transplantation supports the hypothesis that innate inflammation may herald/ influence the acquired response and rejection. Further evidence comes from experimental corneal transplantation in the “high-risk” (vascularised recipient) model which is characterised by increased early infiltration of the graft by neutrophils and macrophages(Yamagami et al., 2005b). In experimental cardiac transplantation in mice, modulation of early innate inflammation in the graft modifies survival. Treatment of graft recipients with the neutrophil-depleting antibody RB6.8C5 significantly improved graft survival(Morita et al., 2001)

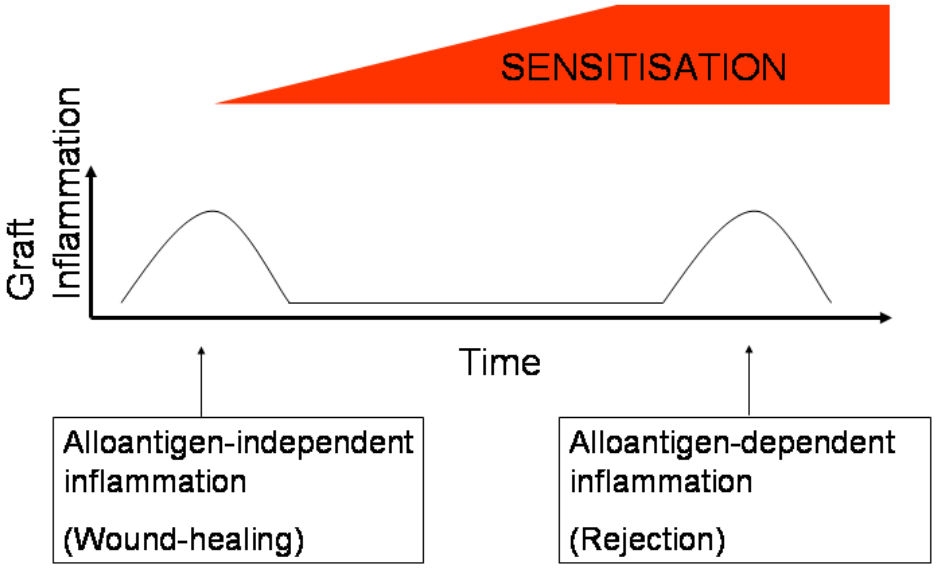


Figure 1.2 A schematic diagram demonstrating the relationship between post-transplant corneal inflammation and time

1.6.3. Sensitisation

The acquired immune response to corneal alloantigen has afferent and efferent components. The afferent limb involves presentation of alloantigen to T lymphocytes. This process, known as sensitisation, is thought to occur in the regional lymph nodes(Yamagami and Dana, 2001). The antigens in question are proteins and peptides derived from donor cells and, in most forms of transplantation, the most potent of these are the Class I and Class II molecules of the Major Histocompatibility Complex. Minor histocompatibility antigens constitute MHC-bound peptides with the unifying property of acting as alloantigens that induce allogeneic tissue rejection. The designation of major- or minor histocompatibility refers to the relative importance of these antigens in

vascularised organ transplants. Interestingly this distinction is less clear cut in corneal transplantation with minor h antigens appearing to be relatively more important. (Sonoda and Streilein, 1992,Sano et al., 1996)

APCs are key cells in sensitisation to alloantigen. Following transplantation the body contains, broadly 2 types of APC: donor APCs in the graft (passenger leukocytes) and recipient APCs in the adjacent tissues. Recipient antigen-presenting cells (APCs) can enter the graft and endocytose exogenous alloantigen. In vascularised grafts entry of recipient APCs occurs via the blood supply. Corneal transplantation rarely involves replacement of the entire cornea. Typically an 8mm button of donor cornea is sutured into a rim of recipient cornea to create a hybrid cornea with donor cells in the centre and recipient cells in the periphery. Recipient APCs in the region of the graft-host interface may pick up donor antigen there or may cross the interface. In any case, once they have phagocytosed antigen, recipient APCs then travel to the local lymph node where the exogenous alloantigen is presented on MHC class II molecules to naïve CD4 cells and on MHC class I to naïve CD8 cells. The exogenous antigen in question could be either a donor minor histocompatibility antigen or part of a donor major histocompatibility antigen(Benichou et al., 1992). One “self” APC activates both CD4 and CD8 cells in what is known as the “three cell model” of alloantigen presentation(Mitchison and O'Malley, 1987). This type of antigen presentation does occur in the transplant setting and is known as **“indirect”** antigen presentation.

There is also another form of antigen presentation which is unique to the transplant setting. Known as **“direct”** antigen presentation, it is mediated by donor APCs(Lechler and Batchelor, 1982). The ability of T-cells to recognise antigenic peptide in association with self-MHC is developed in the thymus by a process of positive selection of self-recognising T lymphocytes. There is considerable experimental evidence that positively-selected T cells can only recognise antigen presented on self –MHC. As such, T cells are said to be self-

restricted. Under the rules of self restriction, alloantigen presented by donor APCs should not be recognised by host T cells if the major histocompatibility antigens are not matched. In reality, the alloantigens *are* recognised by a significant number of host T cells(Detours and Perelson, 2000). Up to 24% of T cells have been found to be capable of reacting with non-self MHC molecules which suggests that self-restriction of T lymphocytes is not an absolute phenomenon. Some of these lymphocytes may recognise, and be primed by, the alloantigenic MHC molecule itself regardless of the peptide it bears(Rogers and Lechler, 2001). One explanation for direct allorecognition may be that certain lymphocytes recognise the foreign MHC +/- protein as self MHC + foreign protein(Rogers and Lechler, 2001).

This method of antigen presentation is consistent with the three-cell model and, in organs other than the cornea, appears to play an important role in acute allograft rejection(Pietra et al., 2000). Directly primed T-cells constitute approx 90% of the alloreactive cells during acute graft rejection in vascularised organ grafts(Benichou et al., 1999,Liu et al., 1993).

It is thought that, for vascularised organ transplants, minimising the direct response may be the first step towards tolerance induction(Jiang et al., 2004). This is consistent with evidence from corneal transplantation which suggests that some of the factors which confer immune privilege do so by minimising or preventing direct antigen presentation. For example, a normal donor cornea contains few mature APCs(Hamrah et al., 2003a) and a normal host cornea contains few lymphatics. Previously published reports confirm that in normal-risk corneal transplantation the indirect route is primarily responsible for sensitisation(Illigens et al., 2002,Kuffova et al., 2008,Boisgerault et al., 2009) Nevertheless, the indirect route of antigen presentation is sufficient to induce sensitisation.

Lechler and co-workers have reported a “semi-direct” pathway of antigen presentation, whereby recipient APCs present whole donor MHC molecules as

well as their own MHC molecules (Herrera et al., 2004) but the possible role of this pathway in corneal transplantation has not been examined.

1.6.4. Clonal expansion

Once a T cell is activated in the lymph node there is rapid clonal expansion of alloantigen–specific T cells which enter the circulation. The lifespan of these cells is limited and it follows that there is a limited window of opportunity for these cells to bring about graft destruction in the absence of continuous antigenic stimulation. The rapid expansion of T-cells is followed by contraction as many effector T cells apoptose(Williams and Bevan, 2007). Memory (central and effector) cells make up part of the T cell repertoire thereafter.
In solid organ grafts the progression from the afferent to the efferent arms of immune response is relentless, resulting in early graft destruction in the absence of systemic immunosuppression. The rejection pattern in corneal grafts is different with apparently tolerated grafts being rejected acutely many years after transplantation. As such the temporal relationship between the afferent and efferent arms of the immune response is less clearcut.

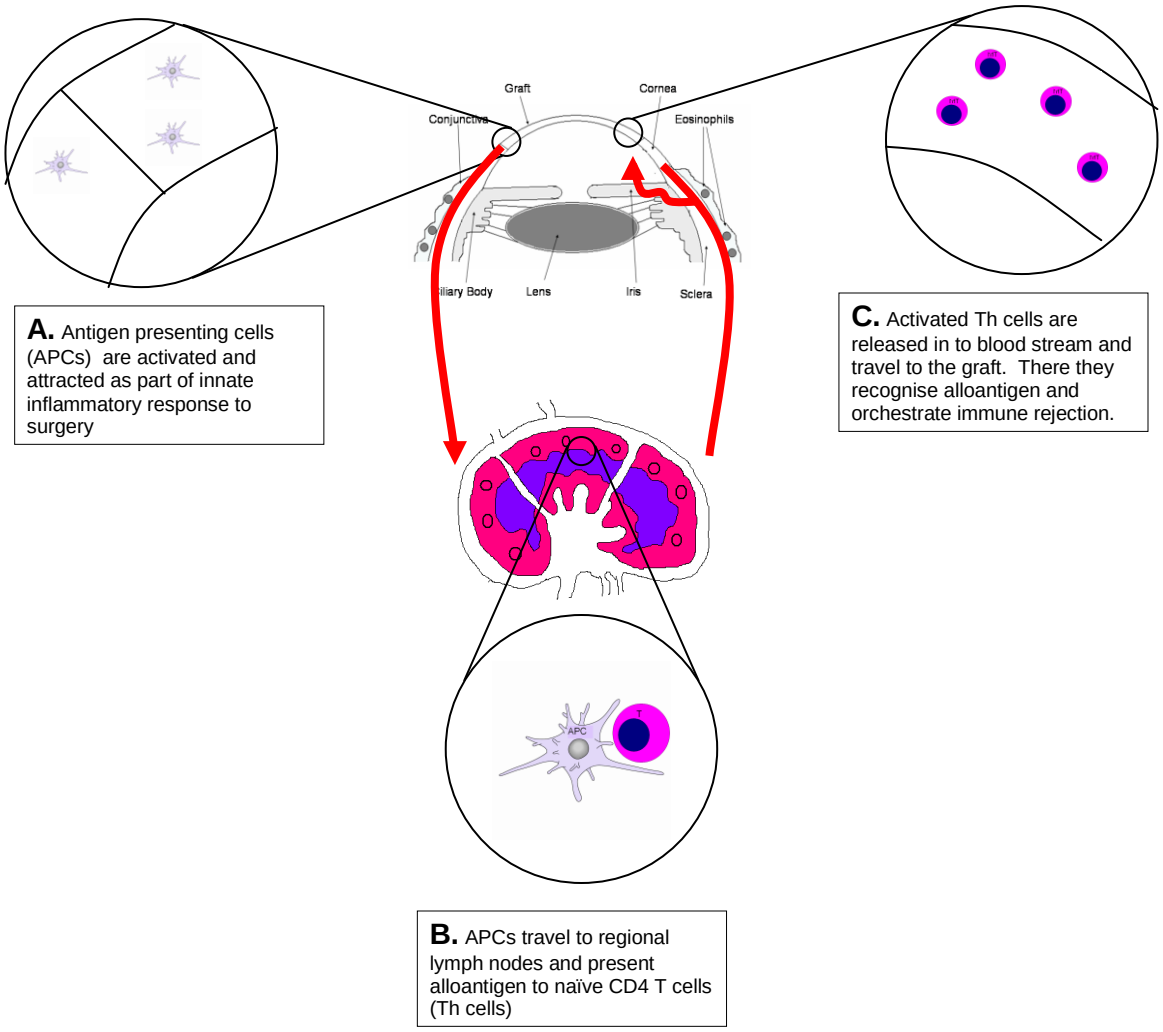


Figure 1.3 A schematic diagram of the immune response to allogeneic donor cornea

1.6.5. Effector Mechanisms: Lymphocytes

Once primed in the regional lymph nodes, activated lymphocytes enter the peripheral circulation. The avascular nature of the cornea and the blood-aqueous barrier provide barriers to immune cell infiltration and endothelial cell destruction. In the case of vascularised corneas immune cells have easier access to graft antigens/cells.

The nature of graft-infiltrating cells in corneal allograft rejection has been studied in human and animal pathological specimens. The cell types which appear in the highest numbers and with the greatest consistency are cells with specific immune capacity such as CD4 cells and CD8 cells, and cells with innate immune capacity such as macrophages and NK cells(Larkin et al., 1997a,Pepose et al., 1985) The presence of a cell in a tissue during rejection does not prove that the cell is causing rejection. The important questions of which cells cause endothelial cell destruction and by what mechanism(s) this occurs remain poorly understood. For instance the mechanisms of allorecognition in the effector stage of graft rejection are unclear.

A particular conundrum has always been the question of how indirectly-primed T cells (host MHC (+mH) molecules) can recognise antigen on donor cells (donor MHC (+mH) molecules). The discovery of the “semi-direct” pathway of antigen presentation, whereby recipient APCs present whole donor MHC molecules as well as their own MHC molecules, provides an explanation for this(Herrera et al., 2004). Kuffova has recently provided convincing evidence of T cell clonal expansion in regional lymph nodes induced by cross-presentation of donor-derived antigen on host APCs.(Kuffova et al., 2008)

The requirement for T cells in graft rejection was established in a number of models of tissue transplantation. Using irradiation and passive transfer of lymphocytes Hall demonstrated that T cells were necessary and sufficient to cause rejection of cardiac allografts(Hall et al., 1978). Because both CD4 and CD8 have been found in pathological specimens of rejected corneal grafts much interest has fallen on the roles of these cells in corneal graft rejection. Convergent studies have demonstrated the presence of 2 distinct lymphocyte populations in response to a corneal allograft. One group appear to be $CD4^+$, IL-2 producing cells which are activated by indirect presentation of alloantigen. The other group are IFN-γ producing $CD8^+$ cytotoxic cells with direct specificity for alloantigen(Boisgerault et al., 2001). T cells may bring about destruction of

other cells either by direct cytotoxicity or indirectly by delayed-type-hypersensitivity (DTH). $CD8^+$ cells act directly on target cells and are cytotoxic but it appears that $CD8^+$ cells are less important in corneal graft rejection than in other organs. In a rat model treatment with anti-CD8 had no effect on corneal graft survival whereas treatment with anti-CD4 reduced the rate of rejection significantly.(Ayliffe et al., 1992) One explanation for this may be that depletion of $CD8^+$ cells with anti-CD8 is insufficient to prevent CD8-mediated rejection (Lee et al., 1994). However the results of experiments with CD4-KO and CD8-KO mice were similar. CD8-KO mice rejected their grafts at the same rate as wild-type mice. CD4-KO mice failed to reject Mh disparate or MHC disparate grafts. They did reject some grafts which were mismatched for Mh and MHC but at a lower rate than wild-type mice(Yamada et al., 1999a,Yamada et al., 2001). While $CD4^+$ cells are capable of using FasL to be directly cytotoxic, their primary modus operandi in corneal graft rejection appears to be via delayed-type hypersensitivity (DTH) by secreting cytokines and recruiting other cells such as macrophages. This is supported by the findings of Joo et al who showed that the DTH response (as measured by footpad swelling in response to injection of alloantigen) rather than the cytotoxic T lymphocyte(CTL) response (as measured by chromium release from labelled donor target cells after in vitro exposure to recipient lymphocytes) was found in rejectors of corneal grafts(Joo et al., 1995). However there appears to be considerable redundancy within the immune reponse to an allograft with several lines of investigation supporting alternative cellular pathways for graft destruction.

Ksander showed that CTL was not induced in low-risk grafts acceptors or rejectors but was induced in high risk grafts all of which rejected(Ksander et al., 1996). Niederkorn's group has shown that high-risk graft rejection may be mediated by $CD8^+$ or $CD8^-$ cells and that, confusingly, this may occur in the absence of systemic DTH or CTL responses(Niederkorn et al., 2006b). They have also recently described CD4 T-cell independent rejection (Niederkorn et al.,

2006a) which is mediated by $CD8^+$ cells or by a novel population of $CD4^-CD8^-$ ("double- negative") cells.
We may conclude from these experiments that $CD4^+$ cells play a more important role in graft rejection than $CD8^+$ cells but that either cell type may mediate rejection and that neither is essential for the process.

1.6.6. Effector Mechanisms: TH1/Th2 balance

CD4(Th) cells mediate their effect by producing cytokines. Depending on the cytokines produced, the response may be classified as a Th1 or Th2 response. Allograft rejection is thought to usually result from a Th1 response(King et al., 2000,Dallman, 1995). Th1 cells produce IFN-γ and IL-2. Th1 mediated inflammation is characterised by the presence of macrophages and is typically seen in delayed-type hypersensitivity reactions. Th2 cells produce IL-4, IL-5 and IL-13. Th2-mediated inflammation is characterised by the presence of eosinophils and is typically seen in allergic reactions or reactions to parasites. Transplant rejection was traditionally thought to be a Th1-driven process as analyses of cytokine production during unmodified rejection of corneal and other grafts showed a Th1 profile(Torres et al., 1996).

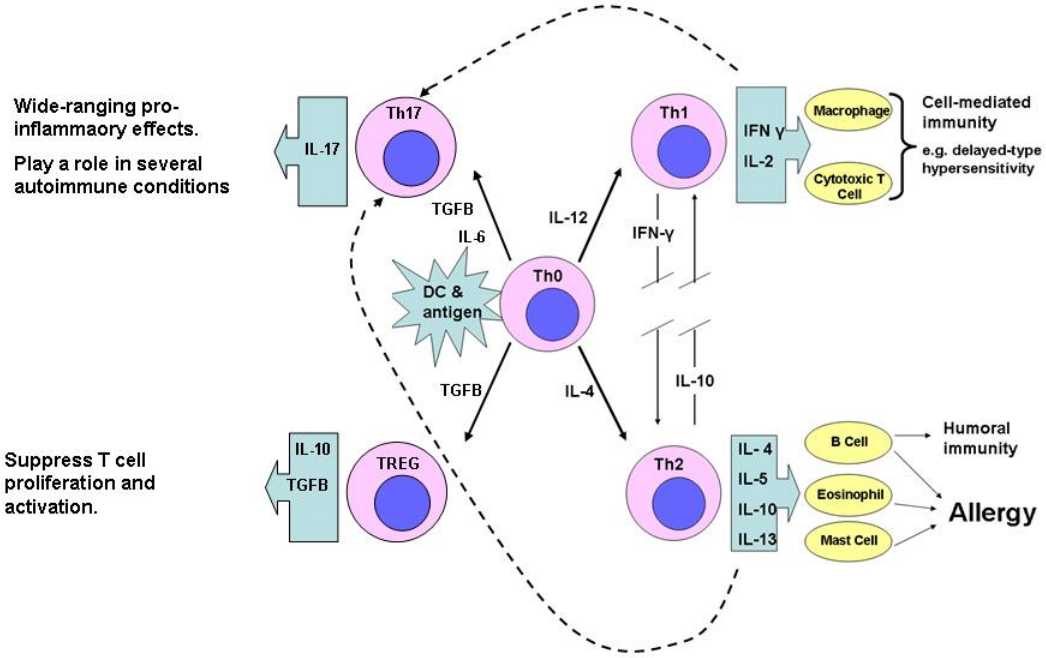


Figure 1.4 A schematic diagram showing induction and roles of Th1 and Th2 cells

The figure also includes other pathways of T cell induction following antigen presentation [Th17 and Regulatory T cells (TREG)] the roles of which remain, as yet, undetermined in corneal allotransplantation.

Chen explored the role of Th2 responses in transplantation by using the mouse model of neonatal tolerance. Mice exposed to donor antigen at the neonatal stage become tolerant of skin allografts later. The allospecific immune responses in the lymph nodes of tolerised mice were characterised by a much higher IL-4 / IFN-γ ratio than controls. This led these authors to believe that inhibition of Th1 responses/ expansion of Th2 responses may be responsible for the induction of neonatal tolerance(Chen and Field, 1995,Chen et al., 1996). This, in turn, led investigators to artificially alter the Th1/Th2 response using various methods in an attempt to induce adult transplant tolerance.

Figure 1.4 shows several of the molecules/ cells that have been shown to be involved in producing a Th1 or Th2 response. Investigators targeted these cells and molecules to induce “Th2 bias” in a variety of tissue transplantation models. Methods used to increase Th2 responses included:

- Inhibition of IL-12(Piccotti et al., 1996)
- Systemic treatment with IL-4(He et al., 1998)
- Gene therapy with IL-4/ IL-10(Furukawa et al., 2005)
- Adaptive transfer of Th2 cells(VanBuskirk et al., 1996,Matesic et al., 1998,Barbara et al., 2000)
- Depletion of host CD8 cells(Chan et al., 1995)
- MHC I matching of grafts (to prevent a CD8 response)(Le Moine et al., 1999b)
- Sensitisation to potent allergen(Yamada et al., 1999b,Beauregard et al., 2005)
- Use of IFN-γ KO mice as recipients(Hargrave et al., 2004,Simeonovic et al., 1999a)

The first direct evidence that Th2 cells could mediate graft rejection came from an experiment by Van Buskirk who transfused Th2 cells to SCID mouse cardiac allograft recipients and observed acute rejection of the grafts(VanBuskirk et al., 1996). There has been no consensus on the question of whether “Th-2 bias” improves graft survival(Piccotti et al., 1997,Tay et al., 2009). As mentioned, neonatal tolerance of skin grafts was associated with increased Th2 responses. Treatment with IL-4 (the key Th2-polarising cytokine) was shown to significantly improve survival of neonatal cardiac allografts in adult mice(He et al., 1998). Combined gene therapy with IL-4 and IL-10 to rabbit hearts before allotransplantation improved graft survival from 7 days to more than 100 days(Furukawa et al., 2005).

Yet it is well known that Th2 responses are potentially harmful eg asthma and that they are capable of inducing graft rejection(VanBuskirk et al., 1996,Matesic et al., 1998). Barbara et al demonstrated that alloreactive Th2 cells were equally

as efficient as Th1 cells at inducing islet cell allograft rejection(Barbara et al., 2000). Promotion of Th2 response via inhibition of IL-12 was shown to accelerate rejection of cardiac grafts(Piccotti et al., 1996). The lack of consensus may reflect the fact that so many different molecular pathways have been used and in so many different tissues and species to induce Th2-bias. Looking at one molecule, IL-4, the effects of post-transplant treatment on graft survival appear openly contradictory. In cardiac transplants in mice and rabbits it appears to prolong survival(He et al., 1998). In rat cardiac transplantation intragraft over expression of IL-4 was found to have no effect on survival(Ritter et al., 1999). In rat hepatic transplants it converts tolerance to rejection(Wang et al., 2005).

In the model of high-risk corneal transplantation, Th2 immune deviation was achieved by repeated exposure to allergen (ova or keyhole limpet hemocyanin). Unsurprisingly, sensitisation with allergen induced a Th2 profile of cytokine production in response to the allergen. Interestingly, sensitisation with allergen also induced a Th2 profile of cytokine production in response to alloantigen. These responses were associated with graft acceptance in high risk eyes(Yamada et al., 1999b). (Of note in this experiment was the fact that the grafts were MHC matched, Minor H Ag mismatched).

Hargrave an co-workers studied corneal transplant rejection in Th2 immune-biased mice using IFN KO mice(Hargrave et al., 2004). Fully mismatched (Major and minor H ag) grafts were first transplanted into both IFN KO and wild-type mice. No difference in graft survival between the two groups was found. Rejection in the “Th2” mice was characterised by a predominant eosinophilic infiltrate while the wild-type “Th1” mice had a predominantly mononuclear infiltrate. The experiment was subsequently repeated using mice mismatched only for MHC antigens but identical at minor h loci. Again no difference in the incidence or tempo of graft rejection between IFNγ-KO mice and wild-type mice as found but their histology differed significantly as before. The experiment was repeated using mice mismatched only for minor h antigens but matched at all MHC loci. Interestingly, in this case, 50% of the grafts were rejected in the wild-

type mice while none were rejected in the IFN KO mice. However, if expression of MHC II on donor corneal Langerhans cells was induced prior to transplantation, 87% of these grafts were rejected in IFN KO mice. Again the histology demonstrated an eosinophilic infiltrate.

These experiments demonstrated that MHC antigens on corneal allografts can be targeted by IFN-γ –independent immune mechanisms that culminate in Th1-independent mechanisms of graft rejection where eosinophils are prominent amongst the graft-infiltrating cells at the effector stage of rejection. It was also shown that, in the absence of MHC II-expressing Langerhans cells, minor-H antigens caused rejection that was IFN-γ- dependent. Hence MHC-matching should help improve survival in TH-2 biased hosts. Or, alternatively, Th2-bias should improve survival in MHC-matched hosts. In their experiments on pancreatic islet cell transplantation, Li et al induced Th2 bias using antiIL-12 monoclonal antibodies and found that this improved graft survival only in MHC-matched grafts(Li et al., 1998).

In their experiments Hargrave et al used BALB/c or BALB/c IFN-γ- deficient mice as the graft recipients and C57BL/6 mice as donors. Recently Yamada reported the results of a similar experiments where the donor-recipient strains were reversed so C57BL/6 mice received BALB/c corneas(Yamada et al., 2009). In these experiments the results were similar to Hargraves' up to a point. Grafts mis-matched for mH and MHC rejected at similar rates in WT and IFN-γ KO animals with eosinohils infiltrating the grafts of IFN-γ KO recipients. In animals mismatched for mH only, rejection rates were also similar in WT and IFN-γ KO animals and no eosinophils were found in rejected grafts.

The important point here is that even if the degree of histoincompatablility is kept constant the immune response to alloantigen is different in different host strains of mice. A more fundamental illustration of this is the fact that in BALB/c to C57BL/6 transplants the rejection rate is over 90% whereas in C57BL/6 to BALB/c recipients the rejection rate is approximately 50%.

Hargraves finding of improved survival of mH-disparate grafts in Th2-biased recipients was in keeping with the findings of an earlier report by Yamada who

also used minor-H antigen mismatched mice to demonstrate that skewing the alloimmune response in a Th2 direction using allergen exposure results in a reduction in corneal graft rejection and suggests that MHC matching improves corneal graft survival in mice with Th2 immune bias.

In Yamada's study Th2 bias was achieved by systemic sensitisation with the allergen ovalbumin(Yamada et al., 1999b). Beauregard et al used a similar technique (using short ragweed pollen rather than ovalbumin) to achieve Th2 bias in their study on corneal transplantation in the setting of allergic conjunctivitis. Corneal graft rejection was accelerated in mice with allergic conjunctivitis and graft rejection in mice with allergic conjunctivitis was characterised by infiltration by eosinophils. A study was designed to distinguish the effects of systemic sensitisation and local conjunctival inflammation on graft survival. The accelerated rate of rejection was concluded to be due to systemic Th2 bias rather than local allergic conjunctival inflammation(Beauregard et al., 2005). This deleterious effect of Th2 bias on corneal graft survival is at odds with the results of Yamada and Hargraves. Yamada used a similar method to induce Th2 bias but his experiments were in high-risk, MHC-matched grafts whereas Beauregards were in normal risk unmatched grafts. In normal-risk recipients, unmatched donor grafts were reported by Hargrave to have unchanged survival but she used a different method of inducing Th-2 bias (IFN-KO) than Beauregard.

1.6.7. Effector Mechanisms: Eosinophils

Whatever the effect on graft survival, where cellular mediators of rejection were studied in these various Th2-biased recipients, rejection was characterised by the presence of Th2 cytokines and graft infiltration by eosinophils (Matesic et al., 1998,Beauregard et al., 2005,Hargrave et al., 2004,Chan et al., 1995,Piccotti et al., 1996,Simeonovic et al., 1999a). It has been proposed that Th2 bias leads to the emergence of alternative effector mechanisms capable of destroying allografts. The effector cells of the Th2 response include eosinophils and these

cells have been implicated in Th2-mediated allograft rejection. Chan was the first to suggest a potential role for eosinophils in graft rejection(Chan et al., 1995). They may do so by secretion of cationic proteins such as major basic protein, eosinophil cationic protein, eosinophil-derived neurotoxin and eosinophil peroxidase. Numerous models of eosinophilic graft rejection in Th2-biased hosts have been published since then. These studies indicate that the relative contributions of alternative effector mechanisms of graft rejection may be dictated by the pre-existing Th1/Th2 bias in the recipient.

Some studies of eosinophilic graft rejection have been characterised by an absence of CD8 cells(Chan et al., 1995,Braun et al., 2000). Activated CD8 cells down-regulate Th2 responses by several mechanisms.

- Secretion of IFN-gamma which has a direct antiproliferative effect on Th2 cells
- Prevention of Th2 polarisation by inducing the production of IL-12 by dendritic cells

Le Moine studied skin grafts in mice of normal genetic background (ie without immune bias) but who were mismatched only at MHC class II. Because the donor-recipient disparity does not involve class I antigens, CD8 cells had no role to play in the alloresponse and the subsequent graft rejection was associated with a dense eosinophilic infiltrate(Le Moine et al., 1999a). This indicates that the relative contributions of alternative effector mechanisms may be dictated by the degree of genetic mismatch of the grafts as well as the host Th1/Th2 balance. A histological study of rejected human corneal grafts has shown a statistically significant increase in the number of eosinophils in grafts rejected by allergic hosts with keratoconus(Hargrave et al., 2003). This raises the possibility that Th2-mediated eosinophilic responses may play a role in graft rejection in these patients and is consistent with the experimental data which has shown eosinophilic infiltration of the cornea during rejection of allografts in Th2-biased hosts.

1.6.8. Effector Mechanisms: Monocyte/macrophage

The heavy mononuclear cell infiltrate which is consistently seen in rejected corneal allografts(Larkin et al., 1997b,Larkin et al., 1997a) is in keeping with a DTH reaction. To investigate the role of macrophages in graft rejection Slegers depleted rat conjunctiva of macrophages using subconjunctival injections of clodronate liposomes. This intervention lead to prevention of graft rejection suggesting that macrophages are necessary for graft rejection(Slegers et al., 2004). The macrophage is known to be an especially multifunctional cell with many roles within both the innate and acquired compartments of immunity. In the context of acquired immunity macrophages may act as APCs in the afferent limb and as effector cells, in conjunction with $CD4^+$ cells in "delayed-type hypersensitivity". Niederkorn's group attempted to separate these roles in corneal graft rejection. They compared allograft survival in 1) nude recipients, 2) nude recipients with adoptive transfer of alloreactive $CD4^+$ cells and 3) nude recipients with adoptive transfer of alloreactive $CD4^+$ cells *and* local depletion of macrophages by subconjunctival injection of clotidronate liposomes. Their results suggest that macrophages are *necessary* as antigen presenting cells rather than as effector cells of graft destruction(Hegde et al., 2005). The precise role of these cells in the effector arm of the rejection process remains unknown.

1.6.9. Effector Mechanisms: Natural Killer cell

Natural killer cells are cells with innate immune capacity which have been found in rejected corneal grafts and more recently, in high numbers, in the aqueous of experimental animals with corneal allograft rejection(Claerhout et al., 2004). These cells usually specialise in the elimination of virally infected cells. The default function of a NK cell is to kill any cell with which it comes in contact. Only the presence of self MHC class I on the cell inhibits this process. In other words, NK cells kill any cell that does not bear self MHC class I (missing self

hypothesis). In vitro studies have demonstrated the capacity of NK cells to kill allogeneic corneal endothelial cells(Claerhout et al., 2004).

1.7. CORNEAL IMMUNE PRIVILEGE

1.7.1. Clinical tolerance v immunological tolerance

True immunological tolerance requires a deviation or muting of the immune response to the alloantigen so that even when the immune system is exposed to the antigen no response is elicited. Renal transplant physicians define tolerance of a graft clinically as stable graft function in the absence of immunosuppression(Girlanda and Kirk, 2007). This type of clinical tolerance is relatively common in low-risk corneal transplantation in both humans and experimental animals and this has led to the misconception that corneal transplantation has been "solved". The fact that corneal grafts reject many years after transplantation during which time they have functioned well without immunosuppression suggests that the tolerance is relative or, at least, that "non-rejection" is not the same as tolerance in the strict sense. Apart from the specific diagnoses of keratoconus and endothelial dystrophy few indications for corneal transplantation can truly be considered low-risk. Nevertheless it is clear that corneal transplants do enjoy a degree of immune privilege. In fact the cornea is an immune-privileged tissue(Hori et al., 2000a) sitting in an immune-privileged site (the anterior chamber of the eye and the avascular peripheral corneal bed).

A number of factors are known to contribute to the relative immune privilege of corneal tissue.

1.7.2. Avascularity of the cornea

Early investigators attributed the immune privilege of the cornea entirely to its lack of vascularity i.e. sequestration of alloantigen from the immune response(Khodadoust and Silverstein, 1972). There is no doubt that this is an important factor. Animal models and multivariate analysis in large human cohort studies have identified corneal vascularisation as the most important factor conferring high-risk status on a corneal graft(Williams et al., 2008).

1.7.3. Lack of mature APCs

In both experimental and clinical transplantation a central button of cornea is used as the graft (rather than the entire cornea). Until relatively recently the central corneal stroma was thought to contain no passenger APCs(Streilein et al., 1979). Recent work by Hamrah has established that it does, in fact, contain APCs but that they are immature and do not express MHC class II in the normal setting.(Hamrah et al., 2002,Hamrah et al., 2003b) Secondly, the normal cornea is devoid of lymphatics to transport APCs. Taken together, these data suggest that, in the low-risk setting, the direct route of antigen presentation would be less important in cornea than in tissues bearing mature APCs grafted to vascular sites. This is confirmed by studies which showed that the indirect route of antigen presentation is more important in corneal graft rejection than in rejection of skin or retinal allografts(Illigens et al., 2002,Boisgerault et al., 2009). This lack of influence of the direct route in corneal graft rejection may explain the following unusual findings:

- In human studies, MHC Class I and Class II matching of corneal grafts has shown no survival benefit (CCTS, 1992).
- In animal studies, mismatches in minor rather than major histocompatiblility antigens have been shown to be more important in influencing graft survival(Sano et al., 1996,Sano et al., 1997).

It is unlikely that the direct route of antigen presentation plays no role at all in corneal graft rejection. Experimental modifications of both the graft and the recipient bed suggest that under certain conditions the direct route of antigen presentation (by passenger APCs) may be important.

A study by Huq et al compared T-cell responses after corneal transplantation to normal (low-risk) and vascularised (high-risk) corneal beds and found directly-primed $CD4^+$ cells in the high-risk but not the low risk setting. In addition, donor APC expression of co-stimulatory molecules was increased in the high risk setting(Huq et al., 2004). Inflammation in the cornea up-regulates MHC class II expression(Donnelly et al., 1990) and the lymphangiogenesis which accompanies vascularisation provides a conduit for egress of APCs(Chen et al., 2004). These data suggest that, in high-risk corneal graft rejection, direct presentation of alloantigen may play a relatively more important sensitising role than in low-risk transplantation (Huq et al., 2004). An obvious therapeutic strategy might be to attempt depletion of passenger leukocytes prior to transplantation. Zhang recently used several methods to do this, achieving a depletion of 39% of passenger leukocytes using anti-CD45 and complement. However this depletion failed to improve longevity of high-risk grafts(Zhang et al., 2009).

Niederkorn studied the role of Langerhans cells, a type of APC found in epithelium, on corneal graft rejection. The central corneal epithelium contains relatively few of these cells. Pre-operative cytokine-induced migration of Langerhans cells into donor central epithelium doubled the incidence of corneal allograft rejection (Niederkorn, 1995). If the increased rate of rejection was truly due to the presence of donor Langerhans cells then there can be only two explanations : 1) the Langerhans cells activate T cells directly or 2) Langerhans cells activate T cells via the indirect/ semi-direct pathway but are particulary immunogenic.

Interestingly pre-operative depletion of donor Langerhans cells does appear to improve graft survival(He and Niederkorn, 1996). It may be that Langerhans cells are more immunogenic than other passenger leukocytes. This is consistent

with the finding that the epithelium is the most immunogenic layer of the cornea(Hori et al., 2000b).

1.7.4. Low MHC expression

The stroma and endothelium have particularly low immunogenicity(Hori et al., 2000b). The endothelium, which is the most important target in rejection, expresses low levels of MHC I and II and high levels of Fas ligand which can induce apoptosis in immune cells and protect the graft (Niederkorn et al., 2006b).

The following factors contribute to the immune privilege of the anterior chamber of the eye:

1.7.5. Blood aqueous barrier

Endothelial cells of the vasculature of the iris and ciliary body have highly selective tight junctions which restrict the movement of cells and proteins between the bloodstream and the aqueous, while still allowing the passage of substances essential to metabolic function (eg oxygen). Under physiological conditions the aqueous does not contain leukocytes but inflammation of the anterior uvea leads to breakdown of the blood-aqueous barrier and extravasation of leukocytes and protein.

1.7.6. Immunosuppresive factors in aqueous humour

The cornea, or at least its endothelium, sits in the anterior chamber of the eye which has been shown to be an immune privileged *site* by Medawar(Medawar,

1948). The aqueous humour which bathes the endothelial cells contains high levels of immunoregulatory cytokines such as TGF-β(Streilein et al., 1992,D'Orazio et al., 1999).

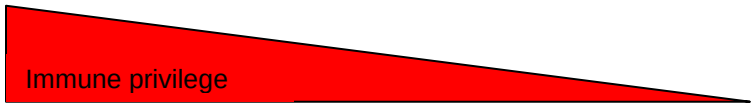
1.7.7. ACAID

In addition, antigen placed in the anterior chamber of the eye alters the immune response (anterior chamber-associated immune deviation or ACAID) to subsequent exposure to the antigen even at a different site(Streilein et al., 1980). Antigen from the anterior chamber leaves the eye via several pathways but at least some leaves via the conventional aqueous outflow pathway and travels to the spleen(Camelo et al., 2005). There the interaction of antigen, NKT cells, B cells and γδ T cells induces a type of relative tolerance(Streilein and Niederkorn, 1981,Sonoda et al., 1999,Skelsey et al., 2001,Skelsey et al., 2003).

1.8. CORNEAL TRANSPLANTATION IN SETTINGS CONFERRING HIGH REJECTION RISK

It is recognised that certain clinical features induce a high risk of corneal graft rejection in humans. These include previous immune-mediated graft failure, corneal vascularisation and ocular inflammation. A mouse model of high-risk corneal transplantation exists whereby vascularisation of the host cornea is induced by placement of corneal sutures prior to transplantation. Comparing the cellular and molecular immune responses in normal and “high-risk” experimental corneal transplantation may be useful in that it may help to identify a “volume control” in some aspect of the immune response to allogeneic tissue, manipulation of which may improve graft survival.

The cellular steps to graft destruction may be summarised as follows:



	Direct route of antigen presentation	Indirect route of antigen presentation	Low-risk	High-risk
1	Egress of Donor APCs	Host APCs infiltrate graft Egress of host APCs bearing antigen Egress of host APC from anterior uvea bearing antigen shed from endothelium	Few APCs ↓MHC II expression Few lymphatics Indirect antigen presentation “Quiet” eye	More APCs ↑MHC II expression Lymphatics Direct antigen presentation Inflamed eye
2	“Direct” Priming of T lymphocytes (*Afferent* Allorecognition)	“Indirect” Priming of T lymphocytes (*Afferent* Allorecognition)	↓MHC II ↓Co-stimulatory molecules ACAID	↑MHC II ↑Co- stimulatory molecules Erosion of ACAID
3	Exposure of circulating primed lymphocytes and other leukocytes to graft	Exposure of circulating primed lymphocytes and other leukocytes to graft	Avascular “Quiet” eye	Vascular Inflamed eye
4	Recognition of alloantigen (*Efferent* Allorecognition)	Recognition of alloantigen (*Efferent* Allorecognition)	↓ MHC expression	↑ MHC expression
5	Recruitment of other effector cells	Recruitment of other effector cells		

Table 1.3 The cellular steps to graft rejection

In a high-risk graft there is an erosion of immune privilege at one or more of these steps. Low-risk grafts that reject later may be thought of as grafts that have acquired ”high-risk” characteristics due to breakdown of immune privilege. In non-vascularised corneas immune rejection occurring months or years after transplantation is often seen to be preceded by an episode of alloantigen-independent inflammation (e.g. loose suture, bacterial infection, viral infection) which may lead to recruitment of immune-competent cells, angiogenesis, lymphangiogenesis and up-regulation of MHC molecules on the graft cells.

Each step and the factors within it contributing to immune privilege are reasonably well understood but the extent to which one step *inevitably* follows the preceding one is less clear. On a fundamental level we may ask of grafts which are not rejected, whether the recipient has not been sensitised due to the immune system not “seeing” the antigen (ignorance of the alloantigen), whether the immune system has seen the antigen but does not or cannot mount a response (tolerance of the antigen) or whether the immune system has seen the antigen and been sensitised but its effector cells cannot see the target antigen due to sequestration of the graft in its avascular bed.
Measurements of ear thickening in response to injected alloantigen are used to measure delayed-type hypersenitvity reactions and, hence, sensitisation following transplantation. Several studies have demonstrated reduced DTH in low-risk grafts compared to high risk and in acceptors of graft compared with rejectors(Sonoda and Streilein, 1993,Yamada et al., 1998). Is this because no antigen has been presented? Dana’s group has demonstrated the presence of donor APCs in the draining lymph nodes hours after transplantation even in low-risk models(Liu et al., 2002). Although the number of these cells entering the lymph node is lower in low- than high-risk recipients their presence suggests that ignorance of alloantigen is unlikely to be absolute. Assuming that some antigen has been presented by APCs in the lymph nodes, does the induction of true immunological tolerance account for the absence of DTH in these cases? If so, we would expect secondary skin grafts to acceptors of corneal grafts to survive

indefinitely. After anterior chamber injection of alloantigen most, but not all, skin grafts do survive(Streilein et al., 1980). In corneal transplantation only the endothelium is in the anterior chamber and egress of antigen/ APCs from the other layers may be via different routes(Camelo et al., 2005). Sonoda showed decreased DTH in acceptors of corneal allografts(Sonoda and Streilein, 1993) but this is unlikely to be the exclusive reason for their non-rejection. In Khodadoust's experiments all acceptors of corneal grafts reject skin grafts from the same donor(Khodadoust and Silverstein, 1972). This is in contrast with what happens following anterior chamber injection of antigen and implies that ACAID induced by corneal transplantation may not be as potent as that induced by intracameral antigen. This may explain the finding that prior intracameral antigen improves corneal allograft survival beyond that of control corneal allografts(Niederkorn and Mellon, 1996). The fate of grafts in pre-sensitised and post-sensitised animals is also interesting. Following corneal transplantation to hosts which have been pre-senitised to alloantigen, some animals reject their grafts and some do not(Sonoda and Streilein, 1992). This tells us that ACAID is not the only phenomenon responsible for immune privilege (ACAID cannot develop in presensitised hosts(Streilein et al., 1980)).

If animals with non-rejected corneal grafts are subsequently sensitised to alloantigen some reject their grafts and some do not(Khodadoust and Silverstein, 1972). This tells us that sequestration of the (avascular) graft from the efferent arm of the immune response is also not the only phenomenon responsible for immune privilege.

While it is tempting to speculate that a single step exists, manipulation of which would induce tolerance or absolute immune privilege in all cases, it is far more likely that the relative contributions to immune privilege at each step is different for each person and for each graft and there is no factor contributing to immune privilege that cannot be overcome by one of the many redundant cellular pathways and mechanisms known to bring about rejection.

1.9. TREATMENT OF ESTABLISHED REJECTION

The mainstay of treatment for established rejection is intensive topical corticosteroid treatment. The most commonly used regimen is prednisolone acetate 1% hourly(Koay et al., 2005,Randleman and Stulting, 2006). This treatment effectively suppresses graft inflammation but once inflammation has been suppressed the question of whether graft clarity will return depends on the extent to which the endothelium has been damaged. Topical steroid has many immunosuppressive effects on immune cells in the cornea chiefly by inducing the expression of anti-inflammatory genes (Annexin-1, SLPI) and repressing the expression of pro-inflammatory genes (cytokines, chemokines, adhesion molecules, MHC molecules)(Barnes, 2006). Many of these anti-inflammatory effects occur in effector cells such as T cells and macrophages but glucocorticoids also affect DC function. They have been shown to alter cytokine production(Toebak et al., 2007), to induce apoptosis in DCs(Brokaw et al., 1998) and to delay DC maturation with resultant impairment of antigen presentation(Piemonti et al., 1999,Rozkova et al., 2006). Corticosteroids also inhibit angiogenesis but this is unlikely to be relevant in setting of acute rejection. Inhibition of IL-2 receptor production inhibits T-cell proliferation but this may not be an important effect of topical treatment as T cell proliferation occurs quite distal to the site of application in the regional lymph nodes. Banerjee et al have demonstrated the development of conjunctival aggregates of leukocytes with the characteristics of conjunctiva-associated lymphoid tissue (CALT) following corneal transplantation in rats(Banerjee et al., 2003) but there has been little investigation into the role of conjunctiva-associated lymphoid tissue (CALT) in corneal transplant rejection. Were clonal expansion of T-cells to occur in the conjunctiva, this would, presumably, be inhibited by topical corticosteroid. A role for CALT would also provide a scientific rationale for the topical use of cyclosporine in treatment and prevention of corneal graft rejection. Some clinicians choose to treat endothelial rejection with systemic as well as topical corticosteroid. However a trial of intravenous methylprednisolone in addition to

intensive topical treatment did not show an improvement in outcome compared with topical treatment alone(Hudde et al., 1999).

1.10. PREVENTION OF REJECTION

The key to minimising immune-mediated graft failure is a dual strategy of (i) identifying patients pre- and post-operatively who are at high rejection risk and tailoring their treatment appropriately and (ii) educating graft recipients as to the signs and symptoms of rejection and how to seek help should these occur.
Pre-operative risk factors for rejection include unmodifiable factors such as a previously rejected ipsilateral graft or previous herpetic keratitis and factors which are modifiable to a greater or lesser degree such as corneal vascularisation or active external eye inflammation. All ocular inflammation should be brought under control where possible before elective corneal transplantation. A degree of regression of corneal vessels may be induced by topical steroid treatment particularly in an inflamed cornea. More established vessels may be difficult to treat. There is a broad spectrum of severity of corneal vascularisation and hence risk in terms of circumference, radial ingrowth and depth of vessels. Corneal surgeons are most concerned about deep vessels and vessels close to the (projected) graft-host interface(Koay et al., 2005).
One rational approach is to management of high rejection risk corneal transplantation is the use of systemic immunosupression with calcineurin inhibitors or sirolimus. Unfortunately there is a lack of high-level evidence favouring any of these treatments which leads to some variablilty in practice.
Long(er)-term local immunosupression with topical corticosteroid appears to be useful in preventing rejection (Nguyen et al., 2007) but the benefit must be weighed against such risks as glaucoma, susceptibility to infection and impaired corneal wound-healing.

1.11. CHEMOKINES AND THEIR RECEPTORS

1.11.1. Chemokine biology

The recruitment of leukocytes from within the vasculature to a focus of inflammation is a multistep process involving adhesion to and passage through vascular endothelium followed by migration across a chemotactic gradient. This process is governed by chemokines, a family of low-molecular-weight polypeptides which bind to G-protein-coupled receptors on leukocytes(Luster, 1998). Chemokines are 8 – 15 kd polypeptides whose primary function is to govern the trafficking of leukocytes. The chemokines are divided into 4 families depending on the arrangement of their cysteine residues at the N-terminus. More than 50 chemokines have been identified. They are classified as belonging to one of four chemokine families: C chemokines, CC chemokines, CXC chemokines and CX_3C chemokines according to their chemical structure (Figure 1.5). The vast majority of which fall into either the CXC chemokine-receptor family or the CC chemokine-receptor family(Ono et al., 2003).

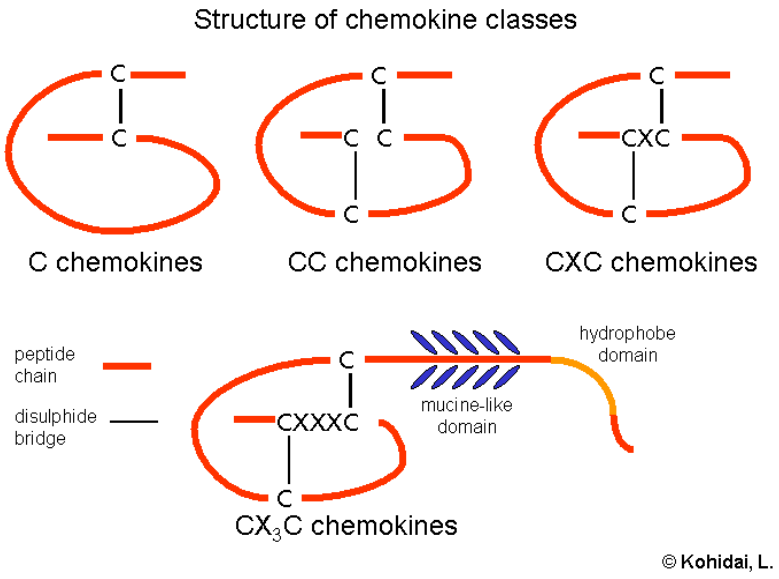


Figure 1.5 The structure of chemokine classes

From Wikipedia www.wikipedia.org

Chemokine receptors are also divided into 4 families according to the ligands they bind (CXC family, C family, CX3C family and CC family)(Roitt and Delves, 2003). Chemokine receptors are polypeptides consisting of 7 transmembrane alpha-helical domains linked to G proteins. Many more chemokine ligands have been discovered than receptors leading to a degree of redundancy/ promiscuity. Chemokine receptors are found almost exclusively on leukocytes with each subset of leukocyte having a characteristic profile of chemokine receptor and ligand expression. Broadly speaking, CC chemokines are chemotactic for T-cells and monocytes and CXC chemokines are chemotactic for neutrophils. This is particularly true of the subset of CXC chemokines which have a characteristic glutamate-leucine-arginine motif near the N terminal of the molecule(Charo and Ransohoff, 2006). (so called ELR+ CXC chemokines, these include IL-8 and KC/ Gro-α). The primary role of chemokines appears to be in coordinating leukocyte movement but they also play a role in leukocyte activation.

Family	Chemokine	Alternative Names
CC	CCL1	I-309/ TCA-3
	CCL2	MCP-1/ MCAF
	CCL3	MIP-1α/ LD78α
	CCL4	MIP-1β
	CCL5	RANTES
	CCL6	C10/MRP-1
	CCL7	MCP-3
	CCL8	MCP-2
	CCL9/10	MRP-2/ CCF18/ MIP-1γ
	CCL11	Eotaxin-1
	CCL12	MCP-5
	CCL13	MCP-4
	CCL14	HCC-1/ HCC-3
	CCL15	HCC-2/ Leukotactin-1
	CCL16	HCC-4/LEC/ LCC-1
	CCL17	TARC
	CCL18	DCCK1/ PARC/ AMAC-1
	CCL19	MIP-3β/ ELC/ Exodus-3
	CCL20	MIP-3α/ LARC/ Exodus-1
	CCL21	6Ckine/ SLC/ Exodus-2
	CCL22	MDC/STCP-1/ ABCD-1
	CCL23	MPIF-1
	CCL24	MPIF-2/ Eotaxin-2
	CCL25	TECK
	CCL26	SCYA26/ Eotaxin-3
	CCL27	CTACK/ ALP/ ESkine
CXC	CXCL1	GROα/ KC
	CXCL2	GROβ
	CXCL3	GROγ
	CXCL4	PF4
	CXCL5	ENA-78
	CXCL6	GCP-2
	CXCL7	NAP-2
	CXCL8	IL-8
	CXCL9	Mig
	CXCL10	IP-10
	CXCL11	I-TAC
	CXCL12	SDF1α/β
	CXCL13	BLC/BCA-1
	CXCL14	BRAC/Bolekine
	CXCL15	Lungkine
C	XCL1	Lymphotactin/ SCM-1α
	XCL2	SCM-1β
CX3C	CX3CL1	Fractalkaline/ Neurotactin

Table 1.4 Classification of chemokines

Because graft destruction is mediated by infiltrating immune cells, interest has fallen on the role of chemokines in this process. Chemokine expression has been studied in various models of transplantation and a wide variety of chemokines are found to be expressed following transplantation. The challenge has been and remains in finding the functional relevance of these chemokines in the transplant setting.

Given that chemokines help to mediate movement and activation of inflammatory cells both the timing and direction of cell movement must be considered in the context of corneal transplantation. Regarding direction, one must consider not only inflammatory cell ingress but also inflammatory cell (APC) egress from the graft. Regarding timing, one must remember that in the early post-operative period there is considerable inflammation in the graft which constitutes a wound-healing (innate) response to surgical trauma. This inflammation is alloantigen-independent. Alloantigen-dependent inflammation occurs later and is responsible for allograft rejection. When studying chemokine expression in transplantation, ideally it is best to try to identify whether the chemokine is primarily involved in cell ingress or egress, in alloantigen-independent or alloantigen-dependent inflammation. These timings are not always possible to separate in practice.

When appraising results of studies of chemokine expression following allotransplantation there are issues of tissue specificity, species specificity and even strain specificity. In addition when looking at kinetics there is the issue of which fixed point to relate to: time of transplantation or time of rejection. With all of these factors to consider it may be too much to expect consistency in the results of studies of chemokine expression during rejection of various transplanted tissues. Nevertheless certain consistent patterns are evident as demonstrated in table 1.5.

	Tissue	Year	Method	Animal	Human	Early	Late
isograft	Skin	1996	NB	Kondo		KC, MCP-1	--
		2000	NB / E	Kondo		KC, MIP-1α, MIP-1β	
		1999	NB	Koga			--
	Heart	1997	NB	Fairchild		MCP-1	--
		2000	RPA / E	Yun		MIP2, MCP1	--
		2001	NB	Morita		KC, MCP-1, MIP-1α, MIP-1β	
		2000	NB	Kapoor		--	
		2001	RT-PCR		Melter		--
	Cornea	2001	RT-PCR	King		RANTES,MCP-1, MIP-1α, MIP-1β	RANTES,MCP-1, MIP-1α, MIP-1β
		1999	RPA	Yamagami			Eotaxin
		2007		Pillai			--
		2006	CBA		Funding		--
allograft	Skin	1996	NB	Kondo		KC, MCP-1, MIP-1α, MIP-1β	RANTES, IP-10
		2000	NB / E	Kondo		KC, MIP-1α, MIP-1β	
		1999	NB	Koga			IP-10, Mig
	Heart	1997	NB	Fairchild		IP-10, MCP-1, and KC	MIP-1α, MIP-1β, RANTES
		2000	RPA/E	Yun		MIP-2, MCP-1	Lymphotactin, RANTES, IP-10
		2001	NB	Morita		KC, MCP-1, MIP-1α, MIP-1β	
		2000	NB	Kapoor		IP-10, Mig	
		2001	RT-PCR		Melter		IP-10
	Cornea	2008	E	Amescua		KC	
		2001	RTPCR	King		RANTES,MCP-1, MIP-1α, MIP-1β	RANTES, MCP-1, MIP-1α, MIP-1β >
		1999	RPA	Yamagami			RANTES, MCP-1, MIP-1α, MIP-1β, MIP-2, IP-10, Eotaxin
		2007	RTPCR	Pillai			IP-10, MIP-2, MIG, MIP-1β, MIP-1α, MCP-1, RANTES, Eotaxin, Lymphotactin, MIP-1γ, Fractalkine
		2006	CBA		Funding		MCP-1,IL-8, MIP-1β

Table 1.5 Chemokine expression following organ transplantation

This table summarises experimental and clinical data on chemokine expression following transplantation of various tissues. Although various techniques were used, there is some consistency in the results. KC, MCP-1, MIP-1α and MIP-1β are seen in the early days post-transplantation. This expression is presumed to be related to alloantigen-independent inflammation. Later, expression of RANTES, MCP-1 and IP-10 are seen. This expression is presumed to be related to alloantigen-dependent inflammation.
NB Northern Blot, E Elisa, RPA ribonuclease protection assay, RTPCR reverse transcriptase polymerase chain reaction, CBA cytometric bead array

1.11.2. Chemokine and chemokine receptor expression in vascularised organ transplantation

Chemokine expression following transplantation has been studied in skin, renal and cardiac allografts. Although there is considerable tissue specificity in the expression of chemokines following transplantation, certain consistent patterns have emerged. Investigators used northern blot and elisa analysis to study chemokine gene expression after tissue transplantation. In studies using mouse models of skin and cardiac transplantation two reasonably consistent patterns of chemokine expression were revealed. Looking first at the early post-operative period (day3) increased expression of KC and MCP-1 in both allograft and isografts was seen in both skin and cardiac grafts(Kondo et al., 1996,Fairchild et al., 1997). In cardiac isografts and allografts increased expression of these chemokines is seen at 6 hours post-transplantation(Morita et al., 2001). By day 8, expression was reduced to normal levels. The fact that expression of these chemokines was similar in allografts and isografts suggests that these they are involved in mediating allo-independent inflammation i.e innate immune responses.

Looking at the timing of the chemokine expression it would appear to correspond to the response to surgical trauma and ischaemia/reperfusion injury. KC and MCP-1 are known to be chemotactic for neutrophils and macrophages respectively. The increased expression of KC and MCP-1 has been shown to correspond to the early post-operative influx of neutrophils and macrophages(Yun et al., 2000).

In the later post-operative period another pattern of chemokine expression emerged whereby increased expression of RANTES and IP-10 was seen in allografts but not in isografts in the days before rejection suggesting a role for these chemokines in alloantigen-dependent inflammation. This pattern of chemokine expression was seen in both cardiac and skin grafts(Kondo et al., 1996,Fairchild et al., 1997,Koga et al., 1999,Koga et al., 2000,Yun et al., 2000). A study of human pathological specimens has also confirmed increased

expression of IP-10 and its receptor CXCR3 in rejected cardiac allografts(Melter et al., 2001). Mig was expressed in skin allografts during rejection and never in isografts(Koga et al., 1999).

MIP-1α and MIP-1β were also seen in skin and cardiac isografts and allografts in the first 2 days after transplantation(Kondo et al., 2000,Morita et al., 2001) but were later found only in cardiac allografts and not skin(Fairchild et al., 1997). In summary the expression of chemokines appears to be bi-modal with an early peak at about 3 days post-transplantation and a later peak in the days immediately prior to rejection. The early peak appears to represent a response to surgical trauma and ischaemia/ reperfusion in vascular organs. The later peak appears to represent graft rejection.

1.11.3. Chemokine and chemokine receptor expression in corneal transplantation

Protein and mRNA findings in corneal allografts and isografts following transplantation are summarised in Table 1.4. King et al studied chemokine expression using RT-PCR in the rat model of corneal transplantation and found that RANTES, MCP-1, MIP-1a and MIP-1b were expressed at similar level in isografts and allografts in the early post-operative period (day 3-7) but that later on (day 9-13) expression was greater in allografts. IP-10 expression was not measured(King et al., 2000).

Yamagami et al studied chemokine expression using RPA comparing isografts, rejected/ rejecting allografts and accepted allografts. For the chemokines Lymphotactin, RANTES, MIP-1α, MIP-1β, MIP-2, IP-10, MCP-1 differential expression was observed in various groups in the order: rejected allograft > accepted allograft> isograft. The greatest difference in expression between accepted and rejected allograft was in IP-10 and RANTES. No TCA-3 expression was seen in any group. Some eotaxin expression was seen in all groups but there was no difference in expression between groups. Interestingly this model used C57BL/6 recipients of Balb/c donors. When this donor-recipient

strain was reversed the results were similar with the exception of IP-10 which was no longer expressed at a high level in rejected allografts. The timing is important here. Eyes were harvested when 50% of allografts had rejected. In this case that was at 3-4 weeks post-transplantation(Yamagami et al., 1999). Pillai used RT-PCR to study chemokine expression in the first 14 days after corneal transplantation. In this model rejection occurred at about day 11 and there was a marked increase over days 7-11 in allografts but not in isografts in the following chemokines: IP-10, RANTES, Eotaxin, MIP-1α, MIP-1β, MCP-1, Lymphotactin, Fractalkine(Pillai et al., 2008a).

An RPA study by Yamagami looked at *early* chemokine expression (i.e. in the first 6 days post-op) after corneal transplantation again in the mouse model. In this case the comparison was not only between isografts and allografts but also "high-risk" allografts. "High-risk" status was conferred by inducing vascularisation of the host cornea prior to transplantation. During the first 6 days post-transplantation expression of RANTES, MCP-1, Mip-1a, Mip-1b, Eotaxin and MIP-2 were equal in isografts and low-risk allografts but was significantly higher in high risk allografts. This increased chemokine expression was associated with higher numbers of infiltrating innate immune cells (macrophages and neutrophils) into high-risk grafts than low-risk grafts at days 3 and 6 post-transplantation(Yamagami et al., 2005b). Amescua et al also studied early chemokine expression in low- and high-risk corneal grafts and found a peak in KC expression in high compared to low-risk grafts at day 3. However unlike Yamagami's study, numbers of graft-infiltrating neutrophils were similar over the first 2 post-operative weeks(Amescua et al., 2008).

The results of these studies are consistent with those in solid organs. The demonstration of no difference between iso and allografts in terms of chemokine expression in the early postoperative period again suggests that this expression relates to alloantigen-independent inflammation. In the cornea this cannot be said to be due to ischaemia/ reperfusion injury as the cornea is avascular. It appears to be due to surgical trauma and wound-healing. Pillai found that

suturing alone caused significant increases in corneal expression of chemokines including Mip-1b, MIP-2, MCP-1, RANTES, IP-10 and eotaxin(Pillai et al., 2008b). Later increased expression in allografts but not isografts is due to rejection or at least an allogeneic immune response. In experimental studies, the timing of this depends on the timing of rejection in a particular strain combination. Funding studied chemokine expression in human aqueous humour during acute endothelial rejection using multiplex bead array technology and found increased level of MCP-1, Mip-1b and CXCL8 in aqueous of eyes with rejecting grafts compared to controls. Finally, there appears to be a difference in chemokine expression kinetics in high- and low- risk corneal grafts(Yamagami et al., 2005b).

1.11.4. Chemokines and APCs

Chemokines and their receptors also govern movements of APCs in health and disease. Chinnery et al found a role for CX3CR1 in the normal recruitment of MHC class II^+ putative DCs to corneal epithelium(Chinnery et al., 2007). Yamagami found a critical role for CCR5 in the recruitment of class II^+ cells to corneal epithelium in response to injury(Yamagami et al., 2005a). The expression of chemokine receptors on DCs depends on their state of maturation(McColl, 2002). Once immature DCs have taken up antigen they begin to express increased levels of CCR7(Dieu et al., 1998) and this appears to play an important role in DC egress via lymphatic vasculature from skin (Saeki et al., 1999) and also from the cornea(Jin et al., 2007). CCL19 and CCL21, ligands for CCR7, are expressed by lymphatic vasculature(Jin et al., 2007).

Chemokine	Expressed by:	Receptor	Found on:
GROα/ KC (CXCL1)	Monocytes, Fibroblasts, Endothelium, Epithelium	CXCR2	Neutrophils, Monocytes
			Endothelium
MCP-1 (CCL2)	Monocytes, Macrophages, Fibroblasts, Endothelium, Epithelium	CCR2	Monocytes, Immature DCs, Memory T cells
MIP-1α (CCL3)	Macrophages, Lymphocytes, Neutrophils, Mast cells, NK cells > epithelium, fibroblasts	CCR1	T cells, Monocytes, Mast cells, Eosinophils, Basophils
		CCR5	T cells, Monocytes
MIP-1β (CCL4)	Macrophages, Lymphocytes, Neutrophils, Mast cells, NK cells > epithelium, fibroblasts	CCR5	T cells, Monocytes
RANTES (CCL5)	T cells, Macrophages, Fibroblasts, Endothelium, Epithelium	CCR1	T cells, Monocytes,
			Eosinophils, Basophils
		CCR3	Eosinophils, Basophils
			Mast cells, Th2 CD4 cells
		CCR5	T cells, Monocytes
IP-10 (CXCL10)	Monocytes, Endothelium, Fibroblasts	CXCR3	Th1 CD4 cells, Mast cells, Mesangial cells
Lymphotactin	CD8 cells	CXCR1	T cells, NK cells
(XCL1)			

Table 1.6 Nomenclature, origin, receptors and target cells of selected chemokines expressed following corneal transplantation

(Charo and Ransohoff, 2006,Roitt and Delves, 2003,Hedrick and Zlotnik, 1998,Deshmane et al., 2009,Levy, 2009,Maurer and von, 2004,Geiser et al., 1993).

1.11.5. Chemokines as therapeutic targets in transplantation

In a model of cardiac allograft rejection, knockout of the CCR1 gene in recipients doubled graft survival(Gao et al., 2000). Graft survival was also prolonged in CCR5 KO recipients of cardiac allografts(Gao et al., 2001). In the same report RANTES KO and MIP-1α KO recipients of cardiac allografts rejected them at a similar rate to WT recipients. RANTES and MIP-1α are the main ligands of both of the receptors CCR1 and CCR5 (both ligands bind to both receptors). These data suggest that if CCR1 and CCR5 play roles in graft rejection, then this role is mediated by binding of ligands other than MIP-1α and RANTES. On the other hand, targeting of the RANTES by an alternative method using anti-chemokine gene therapy (Fleury et al., 2006) has has been shown to prolong cardiac allograft survival.

Similar discrepancies have been found between methods used to target the receptor CX3CR1. Grafting to CX3CR1 KO animals did not improve survival(Haskell et al., 2001) but treatment with anti- CX3CR1 or anti Fractalkine did improve survival of cardiac allografts(Robinson et al., 2000). Treatment with antiserum to MIG was found to prolong survival of skin and cardiac allografts(Koga et al., 1999,Miura et al., 2001).

All of the above targets relate to chemokines found during rejection. Morito also targeted CXCL1, one of the early chemokines found during alloantigen-independent inflammation with antiserum and found that it prolonged survival of cardiac allografts suggesting that modulation of early cellular events in the graft may have far-reaching effects in terms of graft survival(Morita et al., 2001)

Hamrah et al have studied the roles of various chemokines and their receptors using knockout mice and the mouse model of corneal transplantation. Grafts in CCR2-, CCR5-, and Mip-1a KO mice did not show significant improvements in survival but those in CCR1 KO recipients did(Hamrah et al., 2007). Pillai used a viral vector encoding the general chemokine inhibitor vMIPII and found that this significantly improved graft survival(Pillai et al., 2008a).

IP-10 has been found with consistency during rejection of all types of allografts however considerable controversy remains as to its importance and that of its receptor in the rejection process(Halloran and Fairchild, 2008). In particular there appears to be inconsistency regarding the usefulness of CXCR3 and IP-10 as targets to prolong graft survival. Hancock showed that survival of cardiac allografts was improved in CXCR3 KO recipients and in recipients treated with anti-CXCR3 (Hancock et al., 2000). The same group then used anti IP-10 serum and IP-10 KO mice and found that graft survival improved when KO grafts were given to wild type recipients but not when the situation was reversed suggesting that expression of IP-10 from donor cells was more important in the rejection process than in recipient cells(Hancock et al., 2001). More recently there have been conflicting reports which question the potential of pharmacological blockade of CXCR3 in prolonging graft survival(Kwun et al., 2008,Uppaluri et al., 2008,Zerwes et al., 2008). Hamrah found no improvement in survival of corneal grafts in CXCR3- and IP-10 KO recipients(Hamrah et al., 2007).

1.12. LYMPHANGIOGENESIS

1.12.1. History

The thoracic duct was described as long ago as 1650 by Pecquet. It was discovered independently by Rudbeck who published his description of the lymphatic system in 1653. Rudbeck became involved in a bitter dispute with another contemporary anatomist Bartholinus, who was the first to use the term “lymphatics”, over the priority of their findings. Use of dye techniques allowed detailed anatomic delineation of lymphatic vessels but it took hundreds of years before the function of the lymphatics began to be unravelled. In contrast Harvey described the function of the systemic circulation in detail and correctly in 1628.

Several important findings in the late 19th century paved the way for the discipline of immunology:

- Pasteur's popularisation of germ theory
- The beginning of the humoral theory of immunity
- Metchnikoff's discovery of phagocytosis.

Looking retrospectively at these discoveries and considering also i) the well-recognised presence of lymphadenopathy in several infectious diseases and ii) Virchow's (correct) assumption (in 1858) that lymph nodes filter lymph(Virchow, 1975), it seems obvious that lymph nodes would play an important role in immunity. It took until 1935 for lymph nodes to be identified as the site of antibody formation(McMaster and Hudack, 1935) and even that was not accepted for several years(Ehrich and Harris, 1945). It took another 50 years or so (until the discovery of the physiological role of MHC molecules and the T cell receptor) for the afferent limb of the cellular acquired immune response to be relatively fully worked out.

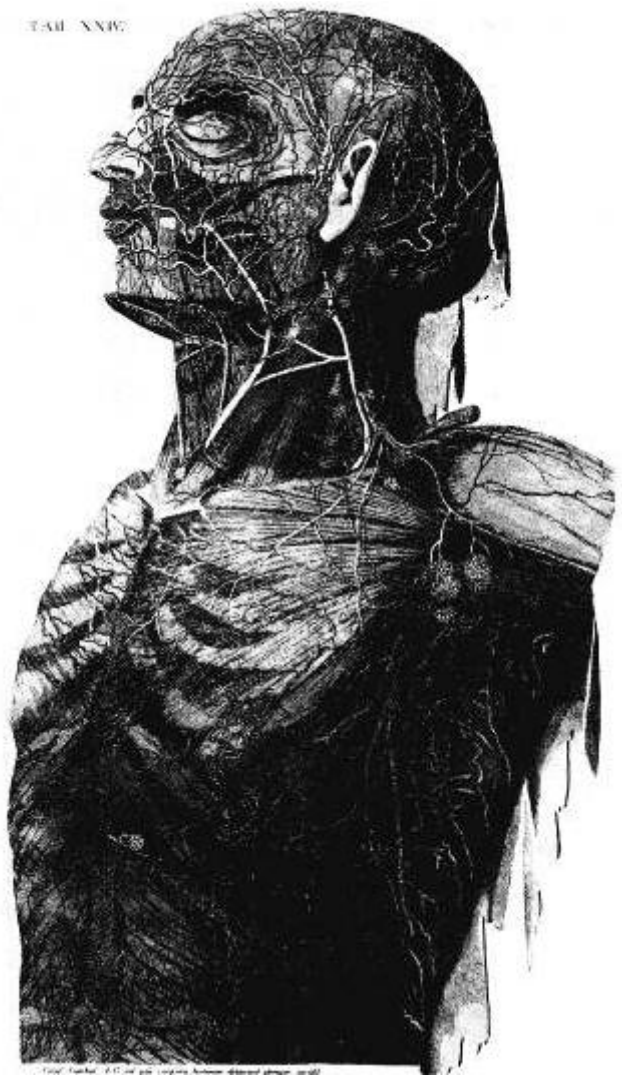


Figure 1.6 Lymphatics of the head and neck

Photograph of an engraving from Mascagni's atlas 1787. (from Kanter MA. Plastic and reconstructive surgery 1987)

In the field of transplantation it was well recognised in the early part of the 20th century that "homografts" of skin did not survive unless they were from genetically identical individuals(Loeb, 1937). However the mechanism of graft destruction remained a mystery and it certainly was not universally thought to be immune-mediated. Holman in 1924 suggested that these grafts may be antigenic but this hypothesis lay fallow for many years(Murray, 1965).

In clinical and experimental studies in the 1940's Medawar showed that the process of graft rejection was immune-mediated(Gibson and Medawar, 1943,Medawar, 1944) but uncertainty remained as to whether it was via cellular or humoral immunity. Typical cellular changes were recorded in histological examination of rejected grafts but debate persisted as to whether these represented cause or effect of rejection. As late as 1942 some prominent surgeons felt that graft infiltrating cells were not involved in graft destruction(Stone, 1942). This issue was clarified in 1954 by Mitchison who transplanted lymph nodes from mice who had rejected skin allografts to naïve mice and conferred adaptive immunity on the naïve animals (whereas serum transfer did not) (Mitchison, 1954). At that stage it was clear that graft rejection was a cell-mediated immune response in which lymph nodes played a part. In the early 1960's two groups demonstrated that excision of the draining lymph node prolonged skin graft survival(Stark et al., 1960,Swartzendruber et al., 1963). In 1967 Hall demonstrated qualitative changes in the cellular content (increased proportion of macrophage-type cells) in the afferent lymphatics in response to a skin allograft(Hall, 1967). In 1968 BIllingham showed the importance of intact afferent lymphatics in skin graft rejection(Barker and Billingham, 1968). In 1970 Collin showed that corneal vascularisation accelerated the delivery of antigen to the draining lymph nodes(Collin, 1970). Looking back this appeared to be a good time to explore the role of afferent lymphatics in the response to allografts more thoroughly but this did not happen (apart from several studies confirming the importance of the draining lymph node in corneal transplant immunology)(Plskova et al., 2004,Yamagami and Dana, 2001).

Possible reasons for this include:

1) The cellular mechanisms of the afferent arm of antigen presentation remained unclear at the time. In 1957 Snell did suggest a role of macrophages in antigen presentation but this was amongst several other putative theories(Snell, 1957). The concept of antigen-presenting cells seems to have been a "slow burner" only gaining acceptance after several lines of

investigation in the 1960s (Mitchison, 1969) and 1970s (Silberberg-Sinakin et al., 1976) and culminating in the discovery of the T Cell Receptor in the1980s(Schwartz, 1985).

2) Several developments at the time (discovery of T cell B cell co-operation, development of the plaque assay and development of the lymphocyte cytolysis Cr51 release assay) made research into the effector mechanisms of lymphocytes more attractive (the “business end” of the cellular immune response)
3) In most circumstances afferent lymphatics are established and unmodifiable, eg skin. The only option to modify these would have been excicion and that may have been deemed impractical.
4) Lymphatics are clear channels containing colourless fluid. Dye techniques used to study lymphatics at this time were technically very challenging. (Nevertheless this did not deter Collin who published extensively on pathological growth of corneal lymphatics in the 1960s and 70s.)(Collin, 1966,Collin, 1970,Collin, 1974)

In early years of 21st century there was a renewed interest in lymphangiogenesis. Possible reasons for this include:

1) During the 70’s /80’s /90’s the function of antigen-presenting cells became better understood, especially after the discovery of the T cell receptor. This brought the afferent part of immune arc into sharper focus.
2) Investment in cancer research facilitated research into afferent lymphatic cell traffic in the context of cancer metastasis.
3) New molecular markers for lymphatic endothelium were discovered. These include VEGFR-3, LYVE-1 and podoplanin.
4) New discoveries were made in the field of VEGF/ angiogenesis eg. VEGFR-3 and its ligands VEGF-C and VEGF-D.

1.12.2. Vascular Endothelial Growth Factor

Lymphangiogenesis occurs in normally developing tissues (except cornea) and in pathological conditions such as inflammation, cancer and wound healing (including cornea). Since their discovery in 1989 the role of vascular endothelial growth factors (VEGFs) in angiogenesis and vasculogenesis of blood vessels has been extensively studied. VEGFs and their receptors also play an important role in lymphangiogenesis.

The VEGF family consists of 5 members: VEGF-A, VEGF-B, VEGF-C, VEGF-D and Placental growth factor. These have different affinities for each of the 3 VEGF receptors: VEGFR-1, VEGFR-2 and VEGFR-3.

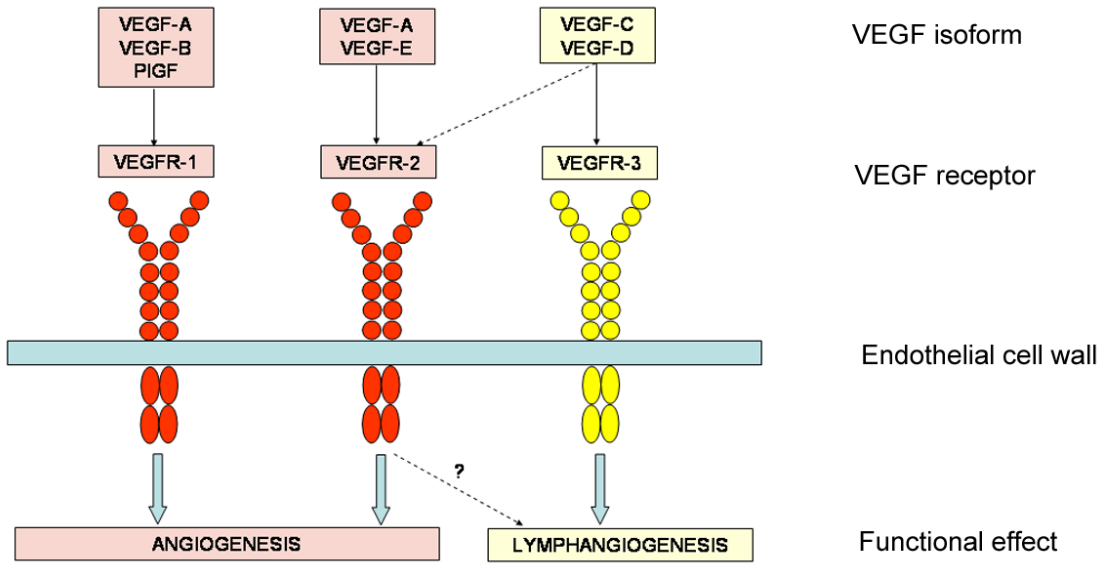


Figure 1.7 VEGF receptors

Traditionally, VEGFR-1 and 2 are found on vascular endothelium and VEGF-R3 is found on lymphatic endothelium. The ligands of VEGFR-3 are VEGF-C and VEGF-D and the VEGF-C/ VEGF-D/ VEGFR-3 axis has been shown to be an essential mediator of lymphatic endothelial cell migration, proliferation and survival(Achen and Stacker, 2008).

More recent evidence shows that VEGFR-2 is found on lymphatic endothelium and may possibly play a role in lynphangiogenesis, possibly by dimerising with VEGFR-3. VEGF-C and VEGF-D are known ligand of VEGFR-3 which induce growth of lymphatic vessels (Karpanen and Alitalo, 2008). Downstream signalling cascades after VEGF receptor ligation remain incompletely understood but several genes have been identified, based on studies in mouse mutants, to be involved in lymphatic vascular development and maturation(Karpanen and Alitalo, 2008).

1.12.3. Corneal lymphangiogenesis

The cornea provides a convenient model for the study of lymphangiogenesis. In 2001 Mimura demonstrated increased expression of VEGF-C and VEGFR-3 accompanying haem- and lymphangiogensis in the rat cornea in the days following injury in a model of corneal neovascularisation(Mimura et al., 2001).
In 2004 Chen showed that inhibition of VEGFR-3 reduced the number of APCs draining from the cornea which in turn reduced DTH reactions and prolonged graft survival. Interestingly it appeared to do so without significantly reducing corneal lymphatic ingrowth(Chen et al., 2004).
Cursiefen showed in 2004 that in normal rejection risk mouse corneal transplantation post –transplantation haemangiogensis is accompanied by lymphangiogenesis. Importantly there was no difference in the rate of both haem- and lymphangiogenesis between allo- and isografts(Cursiefen et al., 2004a). This suggests that post-keratoplasty lymphangiogenesis is alloantigen-independent i.e. mediated by innate immunity. Cursiefen also showed that inhibition of VEGF-A using sequential intraperitoneal injections of an anti-VEGF trap inhibited haem- and lymphangiogenesis and prolonged graft survival. From these data it is not possible to say whether the improved graft survival was due to inhibition of haemangiogenesis or lymphangiogenesis (both were equally inhibited). This finding of a potential role for VEGF-A in lymphangiogenesis was novel and was confirmed in another paper also in which the same authors

showed that suture-induced corneal lymphangiogenesis was accompanied by influx of inflammatory cells (mostly neutrophils but also macrophages which express VEGF-C). VEGF trap inhibited numbers of infiltrating cells after placement of a corneal suture(Cursiefen et al., 2004b) and depletion of macrophages inhibited lymphangiogenesis. The following hypothesis was proposed: Macrophages are recruited to the cornea in response to VEGF-A. These macrophages secrete VEGF-A, VEGF-B and VEGF-C which induce haem- and lymphangiogenesis by binding to their respective receptors on vascular endothelium.

In this hypothesis it is not entirely clear where the original VEGF-A comes from. Studies on the early cellular response to wounding / grafting in all tissues consistently demonstrate an early influx of macrophages and neutrophils(Park and Barbul, 2004). Both cell types have been shown to express VEGF *in vitro* and *in vivo*(Scapini et al., 2004,Lin et al., 2006). Neutrophils entering the cornea in this context have been demonstrated to produce VEGF-A(Edelman et al., 1999).

In a follow-up paper from the same group, Maruyama et al in 2005 provided further evidence that adaptive immunity is not involved in corneal lymphangiogenesis. Their results also showed that corneal lymphatics express CD11b. In their experiment conjunctival macrophages were depleted with cotidronate liposomes and this inhibited suture-induced corneal lymphangiogenesis. Interestingly treatment with clotidronate liposomes also decreased the influx of $CD11b^+$ cells(Maruyama et al., 2005). (One inconsistency in their work is that in this paper the authors identify graft-infiltrating $CD11b^+$ cells as macrophages yet their earlier paper reports that $CD11b^+$ cells in the cornea are predominantly neutrophils(Cursiefen et al., 2004b)). A novel finding reported in this paper by Maruyama et al was that peritoneal macrophages have the capacity to form tube-like structures in vitro(Maruyama et al., 2005) leading the authors to conclude that macrophages may act as progenitor lymphatic

endothelial cells during inflammation. This is supported by the finding of macrophages in the conjunctiva which express the lymphatic endothelial marker LYVE-1(Chen et al., 2005,Xu et al., 2007). There is evidence also in renal transplantation of incorporation of recipient-derived progenitor cells in lymphatic endothelium(Kerjaschki et al., 2006). The precise role(s) of macrophages/ $CD11b^+$ cells in corneal lymphangiogenesis remains unclear. They may act as progenitor cells and may also secrete VEGF-C to induce lymphangiogenesis indirectly.

1.13. ALLERGIC CONJUNCTIVITIS

1.13.1. Atopy

Asthma, eczema and allergic rhinoconjunctivitis are recurrent inflammatory conditions of the lungs and airways, the skin and the mucous membranes respectively. They are known as atopic diseases and tend to occur in individuals with an underlying “atopic” tendency. The precise nature of this tendency remains uncertain. Traditionally atopic inflammation has been thought to be precipitated by exposure to environmental antigens such as pollen. More recently we have become aware of intrinsic genetic determinants which appear to predispose to atopic disease by modifying expression of proteins involved in innate immune defense such as those responsible for the bodies barrier function. The best-described of these is the filaggrin gene in eczema the natural history of which appears to involve considerable gene-environment interaction(Bieber, 2008,van den Oord and Sheikh, 2009).

The prevalence of atopic diseases appear to be increasing(Law et al., 2005) and although several theories have been proposed to try to explain this phenomenon (increased rate of caesarean sections, increased hygiene with decreased exposure to microbes in childhood), none are supported by strong evidence.

1.13.2. General features of allergic conjunctivitis

Allergic diseases of the eye comprise of a number of different inflammatory conditions that share common features such as seasonal variation, association with atopic disease and presumed involvement, to a greater or lesser extent, of the type 1 hypersensitivity mechanism in their pathophysiology. It is traditionally classified into five distinct entities: seasonal allergic conjunctivitis (SAC), perennial allergic conjunctivitis (PAC), vernal keratoconjunctivitis (VKC), atopic keratoconjunctivitis (AKC), and giant papillary conjunctivitis (GPC).

The most common type of allergic eye disease, seasonal allergic conjunctivitis (hay fever conjunctivitis), is also the least serious in terms of visual outcome. Studies of SAC epidemiology using routine data on hospital admissions and primary care consultations are limited by the facts that not all sufferers seek help from healthcare professionals and that sales of “over the counter” hay fever medications are not recorded(Anandan et al., 2006). However it has been estimated that SAC and PAC together account for 98% of allergic eye disease(Ono and Abelson, 2005). AKC and VKC, although much rarer, are more likely to lead to visual impairment, with AKC being the most destructive disease and having the worst visual prognosis.

1.13.3. Seasonal allergic conjunctivitis

Of the allergic eye diseases, SAC represents the most “pure” form of type 1 hypersensitivity. As the name suggests, the symptoms and signs are intermittent and occur rapidly following exposure to a specific allergen, with patients often having a personal or family history of atopy. In the absence of prolonged exposure to allergen, attacks are short-lived. The commonest seasonal allergen is pollen with tree pollen predominating in spring, grass pollen in summer and ragweed pollen in autumn. Symptoms are typically absent during winter. The severity of signs and symptoms vary from patient to patient depending on the specific allergen and the exposure. Patients usually complain of intense itching

of the eyes associated with a watery discharge. The conjunctiva is injected and, in more severe cases, there may be conjunctival chemosis and lid oedema.

1.13.4. Perennial allergic conjunctivitis

PAC is less common than SAC. Although the symptoms and signs of these diseases are the same, the distinction between them lies in the timing of the symptoms. Whereas SAC sufferers have symptoms for a defined period of time, PAC sufferers are sensitive to allergens that are present year-round and so are perennially symptomatic. “Household” allergens such as the dust mite or pet dander are the usual offenders in PAC. These patients may also be sensitive to seasonal allergens and so there may be a superimposed seasonal element to their symptoms.

1.13.5. Atopic keratoconjunctivitis

AKC constitutes a more relentless form of conjunctival inflammation than either SAC or VKC. Atopic dermatitis (eczema), a pruritic skin condition that affects 3% of the population, is present in 95% of patients with AKC(Bielory, 2000). Conversely, 25-40% of atopic dermatitis patients have AKC(Foster and Calonge, 1990).

Typically patients have had atopic dermatitis since childhood with ocular symptoms developing at a later stage. Symptoms may begin in the late teens or early twenties but the peak incidence is between the ages of 30 and 50. Males are more commonly affected than females and there is often a personal or family history of other atopic diseases. Unlike SAC, and most cases of VKC, the symptoms tend to be perennial.

Bilateral itching of the eyelids and periorbital skin is the most frequent symptom. Patients also complain of tearing, photophobia, discharge, burning and blurred

vision. Depending on the severity of corneal involvement, patients may complain of a foreign body sensation and pain.

The periorbital skin typically has the dry, indurated and scaly appearance of eczema. Eyelid swelling may contribute to the generalized wrinkling of the skin. Colonisation of the lid margin with staphylococcus with resultant staphylococcal blepharitis is common(Tuft et al., 1992). There is typically a papillary reaction on the tarsal conjunctiva. The bulbar conjunctiva may show non-specific signs of inflammation such as hyperaemia or chemosis. Rarely, papillary hyperplasia of the limbal conjunctiva occurs resulting in a gelatinous limbal nodule similar to those seen in limbal VKC. Prolonged or severe inflammation may result in conjunctival cicatrisation.

Visual deterioration in AKC is most commonly caused by corneal complications. Corneal scarring in AKC may result from vascularisation, infection or ectasia. A broad spectrum of corneal disease may be seen depending on the severity and chronicity of inflammation. Punctate epithelial erosions are seen early in the course of the disease. The severity of the corneal erosions correlates with the number of inflammatory cells(especially eosinophils) in brush cytology samples from the superior tarsal conjunctiva(Takano et al., 2004). Peripheral corneal vascularisation, which may be associated with opacification, is common. These changes may occur as a result of limbal stem cell deficiency. Rarely, corneal vascularisation may encroach on the visual axis and cause visual impairment. Epithelial erosion may coalesce to form non-infectious corneal ulcers. Toxic granule proteins derived from conjunctival eosinophils have been implicated in the pathogenesis of these ulcers(Messmer et al., 2002).

1.13.6. Vernal Keratoconjunctivitis

A disease of childhood, VKC accounts for 0.5% of allergic eye disease(McGill et al., 1998). Like AKC it has a male preponderance but onset is

much earlier, typically late in the first decade. It is seen most commonly in temperate climates such as those of the Mediterranean, South Africa and North America. There is frequently a personal or family history of atopy but this association is not as strong as in other types of allergic eye disease, with a large proportion of VKC patients having no such history.

In the majority of cases the disease shows seasonal variation with symptoms typically appearing in spring and lasting about six months. Additional recurrences in winter are common. In some cases the disease evolves over time into a more chronic, perennial form of inflammation with up to one quarter of VKC patients having a perennial form of the disease from the outset(Bonini et al., 2000). Although serious visual complications may occur, VKC is a less destructive disease than AKC and usually burns itself out by the early twenties(Leonardi and Secchi, 2003).

Symptoms are usually bilateral but may be asymmetrical and, like all allergic eye diseases, itching is a cardinal feature. Photophobia is also prominent and patients may complain of tearing and a mucoid discharge. Depending on the severity of corneal involvement, they may also complain of a foreign body sensation or pain.

In contrast to AKC, the periorbital skin is usually unaffected. The disease is further classified into tarsal, limbal or mixed VKC depending on the location of the conjunctival inflammatory signs.

In tarsal disease the inflammation is predominantly in the superior tarsal conjunctiva although the bulbar conjunctiva may show non-specific signs such as injection or chemosis. The superior tarsal conjunctiva develops a papillary reaction. Papillae are typically large (>1mm) and diffuse giving a “cobblestone” appearance. These tarsal papillae tend to persist even when the disease is quiescent but become hyperaemic and oedematous during periods of disease activity. The presence of a thick, mucoid, white secretion associated with these papillae is another indicator of disease activity.

Sight-threatening complications occur less frequently in the cornea than in AKC. However, both non-specific and pathognomonic corneal signs are seen. In a

series of 195 patients with VKC, 9.7% developed corneal ulcers and 6% developed a permanent decrease in visual acuity(Bonini et al., 2000). Abnormalities of the central and superior cornea are most commonly seen in tarsal disease. In its earliest form there may be only punctuate epithelial erosions. These may, with time, coalesce to form larger erosions that may in turn evolve into the characteristic “shield” ulcer of VKC.

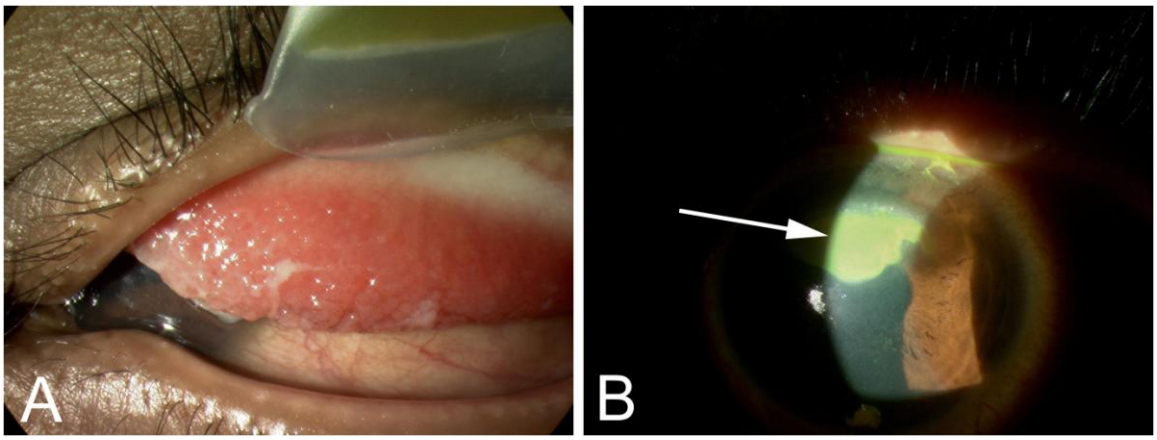


Figure 1.8 Vernal keratoconjunctivitis: Signs of disease activity

Giant papillae are seen on the everted tarsal conjunctiva (A). A macroerosion has formed on the subjacent superior cornea in the same eye (B).

1.13.7. Immunobiology of seasonal allergic conjunctivitis

The most acute form of allergic eye disease— seasonal allergic conjunctivitis—involve a typical mast cell (IgE) mediated Type I hypersensitivity reaction. This reaction occurs in three phases: the sensitization phase, the early phase, and the late phase. The sensitization phase occurs on initial exposure of the ocular surface to aeroallergens. These allergens are phagocytosed by the antigen presenting cells (APCs) on the conjunctival mucosal epithelium. They are processed within the APCs and presented on the surface of these cells as a peptide fragment in association with the major histocompatibility complex (MHC) class II molecule. This allergen/MHC complex on the surface of APCs then interacts with naïve T-helper (Th) cells causing maturation of these naïve cells in

lymphoid tissue to Th2 cells resulting in the production of cytokines which interact with naïve B cells. This causes antibody class switching to occur in these B cells such that they start to produce immunoglobulin E (IgE)(McGill et al., 1998). This IgE binds to the high affinity receptor (FcεRI) on the surface of mast cells and basophils.

When this sensitized eye encounters the same allergen on a subsequent occasion, a Type I hypersensitivity response is triggered as allergen attaches itself to mast cell linked IgE antibodies, causing cross linking of these antibodies. This causes changes in the mast cell outer membrane making the mast cell more permeable to calcium ion with subsequent mobilization of intracellular calcium. When a critical mass of IgE antibodies become cross-linked, this increased permeability causes the mast cells to rupture and degranulate, releasing a variety of primary inflammatory mediators stored in their preformed granules. These include histamine, serotonin, eosinophil and neutrophil chemotactic factors(Leonardi et al., 2007). It is these mediators that are responsible for the symptoms of the early phase response, usually beginning within seconds of subsequent allergen exposure and lasting for up to 40 minutes after exposure. This released histamine binds to receptors on adjacent tissues to cause the classical symptoms of itching, swelling and oedema, and redness. Furthermore, the aggregation of the FcεRI receptors triggers a complex biochemical intracellular cascade involving the metabolism of arachidonic acid from membrane phospholipids into various prostaglandins, thromboxanes and leukotrienes. These factors are the newly formed mast cell mediators, which contribute to the inflammatory reaction by the recruitment of additional inflammatory cells, leading to the late phase reaction.

The late phase reaction begins between 4 to 12 hours after the release of the mast cell mediators(Choi and Bielory, 2008). It peaks at about 24 hours and, in the absence of repeated antigen exposure, settles at about 72 hours(Choi and Bielory, 2008,Li et al., 1996). This response is characterized histologically by

infiltration of the conjunctiva by neutrophils, basophils, eosinophils ,lymphocytes and macrophages(Leonardi et al., 1992,Li et al., 1996,Bacon et al., 2000,Choi and Bielory, 2008)—these all serve to amplify the conjunctival mucosal inflammatory reaction.

More chronic forms of allergic conjunctivitis are characterized by cellular infiltrates (T lymphocytes, macrophages) which are less typical of a clear-cut Type 1 hypersensitivity response and are more suggestive of a DTH response(Metz et al., 1996).

1.13.8. Pharmacotherapy of Allergic conjunvtivitis

1.13.8.1. Antihistamines

Antihistamines work by binding to the histamine (H) receptors thus preventing the action of this powerful mediator. Although there are four known distinct histamine receptors, the phrase ‘anti-histamine’ as used in the pharmacotherapy of allergic eye disease refers to antagonists of the H1 receptor. Histamine, through its action on these receptors, plays a key role in allergic inflammation. H1 receptor antagonists may be classified according to their chemical class (e.g. piperazines, piperadines etc.) but are more usefully categorised as either sedating- or non-sedating antihistamines. In general, older or ‘first-generation’ H1 receptor antagonists such as chlorpheniramine or cyclizine are sedating while newer ‘second-generation’ agents are non-sedating.

Second generation H1 receptor antagonists (non-sedating antihistamines) are used for the topical treatment of the benign forms of allergic conjunctivitis (SAC and PAC), and these include levocabastine, azelastine and emedastine. They all bind selectively to H1 receptors in the conjunctiva, have little or no effect on dopaminergic, adrenergic or sertotoninergic receptors and have been shown

to be effective at relieving symptoms of allergic conjunctivitis(Solomon et al., 2001,Bielory et al., 2005)..

1.13.8.2. Mast Cell Stabilizers

This group includes the compounds sodium cromoglygate, lodozamide, ketotifen, nedocromil sodium and the more recently introduced olopatadine. Mast cell stabilizers are effective in both mild and severe form of allergic eye disease and have the advantage of having very few side effects, either locally or systemically. However, for patients to receive long-term benefit from them such that expected exposure to allergen reduces the tryptase and inflammatory cells after allergen challenge, treatment is needed for many years(Solomon et al., 2001).

Sodium cromoglygate is the prototypic mast cell secretion inhibitor—it is the oldest and most widely used agent of this family of drugs. However despite its extensive use, the mechanisms of its action remain elusive. Nedocromil sodium can prevent immune responses, such as mast cell degranulation, and this may be due to its ability to inhibit chloride ion flux in mast cells, epithelial cells and neurons. An alternative mechanism of this action may be by the inhibition of IgE production by B cells(Solomon et al., 2001) .

1.13.8.3. Dual-acting Agents

Dual-acting agents are the newest generation of antiallergic agents and are named for their antihistamine effects and their inhibition of mediator release. They offer the advantage of rapid relief of symptoms, produced by immediate histamine receptor antagonism, coupled with the long-term disease modifying benefits of mast cell stabilization. The drugs in this category include olopatadine and ketotifen. Olopatadine hydrochloride is a selective H1 receptor antagonist and inhibitor of mast cell degranulation, preventing the release of histamine and other mediators of the allergic immune response(Bielory, 2002). Studies have

shown it to be significantly more effective than placebo in relieving the itchiness and redness of ocular allergy for up to 8 hours(Abelson, 1998). It is an effective treatment for SAC and PAC and has the advantage of not only being well-tolerated by the patient, but also only requiring twice-daily dosing.

1.13.8.4. Corticosteroids

Topical steroids preparations are the most effective therapy for use in moderate to severe forms of allergic eye disease. However long-term use is associated with an increased risk of the development of cataracts and glaucoma and can potentiate ocular herpetic infections. In fact, topical steroids are responsible for the 2% incidence of glaucoma in VKC patients(Bonini et al., 2004). They work by inhibiting phospholipase A2, an enzyme essential in the synthesis of the prostaglandins. They are also able to inhibit the degranulation of mast cells and basophils, and histamine synthesis. In T cell dependent AKC and VKC, sodium cromoglycate has been used either prophylactically or as maintenance therapy to control mild symptoms only, but is ineffective in acute exacerbations. In acute exacerbations, even the newer class of mast cell stabilizers may not be enough and under these circumstances, steroids (dexamethasone) tends to be used in doses of up to one drop hourly especially if a keratopathy is present (McGill et al., 1998).

1.13.8.5. Calcineurin Inhibitors

The calcineurin inhibitors, cyclosporin A and tacrolimus, are used to induce systemic immunosuppression following organ transplantation. The enzyme calcineurin plays an important role in T cell receptor signalling following antigen presentation. Cyclosporin A and tacrolimus inactivate calcineurin, thereby inhibiting IL-2 production and T cell activation(Denton et al., 1999). In addition, cyclosporine A inhibits histamine release from mast cells and basophils(Bonini et

al., 2004). Systemic cyclosporine A has been shown to improve symptoms in severe AKC(Hoang-Xuan et al., 1997) but its use is associated with potentially life-threatening side effects, such as renal failure, which must be taken into account when considering its use in non life-threatening diseases.

Topical preparations of cyclosporine A are used to try to reduce the requirement for topical steroid in severe allergic eye disease(Donnenfeld and Pflugfelder, 2009). Numerous studies over the past 20 years have shown topical cyclosporine to be effective at reducing symptoms and signs of steroid-dependent AKC. Preparations used include a 2% ointment(Hingorani et al., 1998) and a 0.05% drop(Akpek et al., 2004). Topical cyclosporine has also bee effective in reducing symptoms of VKC (BenEzra et al., 1988,Secchi et al., 1990).

Tacrolimus has been approved for topical use in atopic dermatitis. It is available as an ointment in two strengths 0.1% and 0.03% and there are reports of its use in severe allergic eye disease(Attas-Fox et al., 2008,Joseph et al., 2005).

1.13.9. Allergic conjunctivitis and human penetrating keratoplasty

Ocular inflammation is a recognised risk factor for corneal graft rejection. The phrase ocular inflammation, however, covers a broad spectrum of disease severity ranging from blepharitis to autoimmune disease-induced corneal melting. Although the conjunctival inflammation during an attack may be quite severe, seasonal allergic conjunctivitis is generally considered to lie towards the milder end of the spectrum of ocular inflammation. This is probably because it occurs relatively infrequently, lasts a relatively short time, is often self-limiting and has an excellent visual prognosis. The effect of seasonal allergic conjunctivitis on human corneal graft survival is unknown. Many patients with seasonal allergic conjunctivitis are atopic. Systemic atopy has many effects on the immune system and the effects of these, in turn, on human corneal graft survival are also unclear. SAC is often a co-morbidity for patients with keratoconus yet when

graft survival is stratified according to the indication for transplantation, recipients with keratoconus have the best survival probability(Williams et al., 2008).

Mahmood reported outcomes of a non-comparative series of corneal grafts in recipients with known histories of VKC and concluded that visual outcomes in these patients were good and post-operative complications were low(Mahmood and Wagoner, 2000).

An additional problem in studying the effect(s) of allergic eye disease on human corneal transplantation is the fact that most surgeons increase the intensity and length of post-operative topical steroid treatment in patients with a history of severe allergic eye disease (especially VKC or AKC).

This is borne out by the findings of Egrilmez et al who reported good outcomes in graft recipients with VKC but found an increase in post-operative complications such as premature suture loosening and steroid-related cataract(Egrilmez et al., 2004). On the other hand Wagoner compared outcomes after penetrating keratoplasty in patients with (n=80) and without (n=384) VKC and found no significant differences in either survival or post-operative complications.(Wagoner and Ba-Abbad, 2009).

Investigators suggest a poor prognosis in graft recipients with AKC yet comparative data are hard to find(Easty et al., 1975,Ghoraishi et al., 1995).

There are no human data available on the effect of seasonal allergic conjunctivitis on corneal transplant survival.

Patients with allergic conjunctivitis suffer not only from the local effect of allergic inflammation but also have underlying genetic, structural and immunological tendencies towards atopy which may also, in theory, influence the immune response to transplanted tissue. The finding that graft survival was no different in recipients with or without VKC suggests that the net effects of local conjunctival changes and systemic atopic tendency on the immune response to the graft were not significant. These data are open to other interpretations. It is possible that the local effects and systemic causes of VKC work in opposite

directions with one counterbalancing the other. It is also possible that increased frequency/ length of topical steroid treatment in recipients with VKC masked a potential effect on graft survival.

Kirkness looked at corneal transplant outcomes in patients with keratoconus and found no difference in survival in those with (28%) and without atopy(Kirkness et al., 1990). These data seem to suggest that even the associated systemic atopy does not influence graft survival in allergic conjunctivitis. On the other hand, Cursiefen has shown that graft survival is shorter in corneal graft recipients with atopic dermatitis(Nguyen et al., 2008) and that this effect appears to be independent of the history of allergic eye disease. These data would support the hypothesis that accelerated corneal graft rejection in recipients with atopic disease is due to systemic features of atopy and their effects on the immune response. This hypothesis is also supported by the experimental work of Niederkorn who reported accelerated corneal graft rejection in experimental animals with allergic airways disease(Niederkorn et al., 2009).

It is difficult to study “allergic eye disease” in humans as a risk factor for corneal graft rejection because this diagnosis includes at least 3 different diseases: SAC, VKC, and AKC. In addition, in each case the disease may be active or quiescent which gives, in theory, at least 6 different clinical scenarios. An elective transplant would rarely or never be perfromed in an eye with active disease. Quiescence either occurs naturally or is pharmacologically induced prior to surgery. Accordingly, data from human corneal transplantation are unlikely to be sufficient to differentiate the effects of active (in-season) allergic eye disease and quiescent disease.

In summary, the problems with trying to study effects of allergic eye disease in humans include:

- Difficulty in measuring the prevalence of allergic eye disease amongst transplant recipients especially those with less severe disease.
- Surgeons’ tendency to wait until quiescence of conjunctival inflammation has been induced (either pharmacologically or naturally) before operating.

- Surgeons' tendency to prescribe extra topical steroid in graft recipients with allergic eye disease.

An important and interesting phenomenon seen in atopic recipients of corneal grafts is that of atopic sclerokeratitis(Lyons et al., 1990). This condition is characterised by severe post-operative scleral and corneal inflammation with "cheese-wiring" of the corneal sutures. This condition, which becomes evident within 3 to 4 weeks of transplantation, usually requires systemic immunosuppression to maintain a healthy graft.

1.14. AIMS

My original aims were:

- To investigate the effect of perioperative allergic conjunctivitis on corneal allograft survival and infiltrating cells during graft rejection
- To characterize the phenotype of inflammatory cells in human aqueous during acute corneal allograft endothelial rejection in naïve and atopic recipients of corneal allografts.

Early results from the experiments carried out to address these aims allowed generation of new hypotheses resulting in additional aims. These included:

- To investigate the effect of perioperative allergic conjunctivitis on chemokine expression during graft rejection
- To measure chemokine and cytokine expression in human aqueous during corneal allograft rejection
- To investigate the effect of perioperative allergic conjunctivitis on early post-keratoplasty corneal inflammation and lymphangiogenesis

2. CHAPTER 2: General descriptions of the methods used in these experiments including some discussion on optimisation of techniques

2.1. Introduction

This chapter contains detailed descriptions of the individual techniques used in my experiments. These include descriptions of how the techniques were optimized. Description of methodology in later chapters concentrates mostly on experimental design and the reader is referred to this chapter for detailed description of the individual techniques.

2.2. CORNEAL TRANSPLANTATION

2.2.1. General considerations

The mouse model of corneal transplantation has been in use by several groups over the past two decades(Sonoda and Streilein, 1992,Ardjomand et al., 2003,He and Niederkorn, 1996). The advantages of a mouse model over other larger animals are:

- Mice are less expensive to buy and keep than larger animals.
- A wide range of reagents are available for analyzing murine tissue and cells.
- Many inbred strains of mice are available which allow control of histocompatibility in transplants.
- Many mice with specific genes “knocked-out” are available allowing study of the specific role of certain proteins in transplantation.

The disadvantages of the mouse model are:

- The procedure is technically difficult
- The diagnosis of rejection is based on the loss of graft clarity and is subjective.

2.2.2. Albino Host: Animals

Female 6-8 week old A/J strain mice (H-2^k; Harlan UK, Bicester, UK) were used in the allergic conjunctivitis induction protocol and subsequently as corneal allograft recipients. Adult female C57BL/6 strain (H-2^b; Harlan UK, Bicester, UK), which provide a full MHC mismatch and multiple minor mismatches, were used as donors.

Albino mice have 2 important advantages over pigmented mice as recipients in the mouse model of corneal transplantation:

1. The visible pattern of iris vessels allows easier grading of corneal clarity (see figures 2.1 & 2.2)
2. In my hands, the surgery is technically easier and results are better in albino than pigmented recipients

2.2.3. Albino Host: Surgical technique

To dilate the pupil, graft recipients received an intraperitoneal injection of 50µl of atropine sulphate (10mg/ml) and cyclopentolate 1% and phenylephrine 2.5% eyedrops 15 minutes prior to surgery. Donor mice were sacrificed and their eyes enucleated and placed in a Petri-dish containing sterile PBS. The eye was held underwater using a micro notched forceps (Duckworth and Kent, Baldock,UK) and the epithelium and superficial stroma of the cornea was scored concentric with the limbus using a 2.5mm corneal trephine (Geuder, Heidelberg, Germany). Fixation of the donor eye under water was made easier by taping a thin strip of Styrofoam (approximately 4mm thick) to the base of the Petri-dish. Removal of a disc of Styrofoam using the 2.5mm trephine provided a “divot” into which the eye could sit, allowing easier manipulation. Using a 21G needle the anterior chamber was entered at a point along the score on the cornea. Using this entry site as a starting point, the donor corneal button was excised along the score using curved microscissors. This procedure was performed while keeping the donor cornea

under PBS at all times. The donor cornea button remained in PBS while the recipient bed was prepared.

Recipient mice were anaesthetized with intraperitoneal injection of fentanyl fluanisone and midazolam. The anaesthetic solution was drawn up containing 1 part Midazolam (2 mg/ml), 1 part Hypnorm and 2 parts water for injection. The resulting mixture contained 0.5 mg/ml midazolam, 2.5 mg/ml fluanisone and 0.079 mg/ml fentanyl citrate and was administered as a single intraperitoneal injection of 0.15ml (or 10ml/kg body weight approximately). Once anaesthetized, the mouse was positioned lying on its side with its head resting on a small block of Styrofoam so that the eye receiving the graft faced directly upwards. With the mouse in this position a drop of sterile PBS placed on the mouse eye would remain sitting over the eye creating a “bubble” through which the rest of the operation was performed. This “bubble” technique prevents drying of the recipient lens following removal of the recipient cornea and prevents cataract formation. This technique also maintains the submersion of donor endothelium in PBS while the graft is being sutured in place and has a protective effect on the endothelium.

The recipient epithelium and superficial stroma of the cornea was scored concentric with the limbus using a 2.0mm corneal trephine (Geuder, Heidelberg, Germany). Using a 21G needle the anterior chamber was entered at a point along the score on the cornea. Using this entry site as a starting point, the recipient corneal button was excised along the score using curved microscissors. The donor button was next transferred from the Petri dish into the “bubble” of PBS overlying the recipient eye. 11-0 nylon suture on a 3.8mm 3/8 circle taper point needle was used to suture the graft to the host cornea. To secure the graft a first suture was tied with a releasable knot. This suture was not cut but used to secure the graft as a continuous suture with typically 10 bites (Figure 2.1). Once the final bite was taken the original knot was released and the ends tied securely. It was not possible to bury the knot in the thin mouse cornea.

At the end of the procedure chloramphenicol ointment was applied and a blephorraphy was performed using 7-0 vicryl suture (Ethicon). This was opened after 48 hours and, at this stage, eyes with infection, haemorrhage, cataract, significant anterior synechiae or iris prolapse through the wound were excluded. Thereafter, the eyes were examined three times weekly under brief inhalational isoflurane anaesthesia and the graft graded as described below. Corneal sutures were removed at 7 days. Mice were placed in an anaesthetic chamber. The isoflurane vaporiser was set at 2% with an oxygen flow rate of 2L/min. Once the mice lost their righting reflex they were removed from the chamber and a drop of proxymetacaine was placed on the cornea before removal of sutures.

2.2.4. Albino Host: Grading system

The following corneal opacity grading system was used. This system has previously been described and used in experimental studies of corneal transplantation.

0: Completely transparent cornea

1; Minimal corneal opacity, but iris vessels easily visible

2: Moderate corneal opacity, iris vessels still visible

3: Moderate corneal opacity, only pupil margin is visible

4: Complete corneal opacity, pupil not visible

Corneal graft rejection was diagnosed when the corneal clarity score increased to 3 in a graft which was previously transparent following surgery (Figure 2.2).

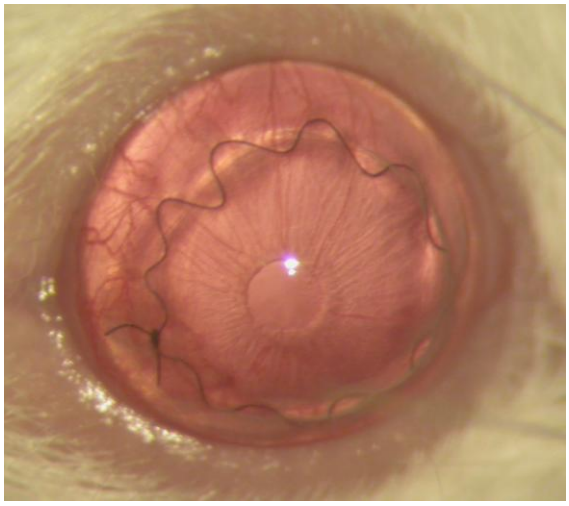


Figure 2.1 A mouse corneal transplant at 1 week post-surgery with suture in situ

The pupil is central and circular indicating an absence of wound synechiae.

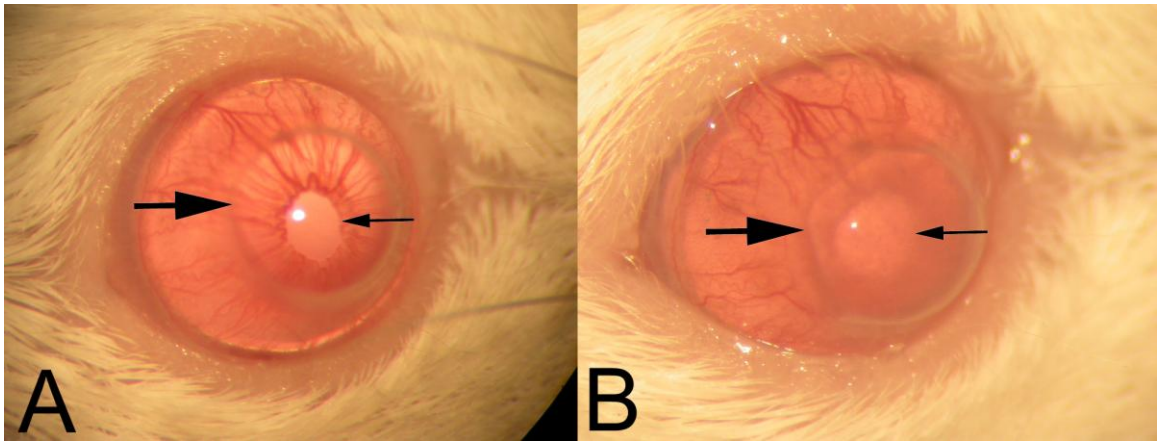


Figure 2.2 Diagnosis of corneal transplant rejection in albino mice

A. A transparent graft. The graft and pupil margins are indicated by large and small arrows respectively. Iris vessels are easily seen through the transplant (grade 0). **B.** A rejected graft. Iris vessels are no longer discernible through the graft although the pupil margin (small arrow) can be identified (grade 3).

2.2.5. *In vivo* measurement of corneal thickness

Measurements of central graft thickness were taken using the Corneogage pachymeter (Sonogage, Cleveland, Ohio) in the donor cornea prior to excision and again immediately post-transplantation. Further measurements were taken under brief inhalational anaesthesia on post-operative day 2 and on alternate days thereafter. Mice were placed in an anaesthetic chamber. The isoflurane vaporiser was set at 2% with an oxygen flow rate of 2L/min. Once the mice lost their righting reflex they were removed from the chamber and a drop of proxymetacaine was placed on the cornea. The Corneogage pachymeter probe was placed on the centre of the graft in a gentle dabbing motion until a reading was recorded. For each measurement three readings were recorded and the average calculated. Repeat measurements were taken by the same examiner or by a second examiner to assess intra-observer and inter-observer variability respectively.

2.2.6. Pigmented host: Animals

As mentioned, albino animals are preferrable as graft recipients. However, pigmented mice must be used as recipients in certain circumstances. For instance, mice with a specific gene knockout of interest may only be available on a pigmented background.

Female 6-8 week old C57BL/6 strain (H-2^b; Harlan UK, Bicester, UK) were used as corneal allograft recipients. Adult female Balb/c strain (H-2^d; Harlan UK, Bicester, UK), which provide a full MHC mismatch and multiple minor mismatches, were used as donors.

2.2.7. Pigmented Host: Surgical technique

Once proficiency was gained in transplanting to albino mice, grafts were attempted in pigmented recipients. As before, isografts were performed to differentiate surgical failure from rejection. Interestingly when using the same technique as used in albino recipients (with 90% success) the success rate was 0%. Grafts in pigmented recipients became opaque in the early days after transplantation and failed to clear. Significant anterior synechiae were usually present (Figure 2.3). The reason(s) for this are not clear. Certainly the iris in pigmented animals appeared during surgery and on histology of normal eyes (Figure 2.4) to be a thicker and more substantial structure than that in albinos. The increased thickness of the pigmented iris may have contributed to synechiae formation by bringing the anterior surface of the iris closer to the corneal endothelium. (Mice have relatively shallow anterior chambers due to the relatively large crystalline lens). It is also possible that the melanocytes in pigmented irides contribute somehow to increase the “stickiness” of the iris. Whatever the cause for the anterior synechiae it was felt that these may be causing the surgical graft failure, so the surgical technique was modified as follows to try to prevent or at least minimise synechiae formation:

A smaller diameter 2.0mm donor button was sutured into a 1.5 mm recipient corneal bed with a continuous 11-0 nylon suture (instead of a 2.5mm button into a 2mm bed). At the end of the procedure a tarsorraphy was performed. This was opened after 24 hours and a drop of pilocarpine 1% was instilled. Thereafter, the eyes were examined three times weekly under brief inhalational isoflurane anaesthesia and the graft graded. Corneal sutures were removed at 7 days post-transplantation.

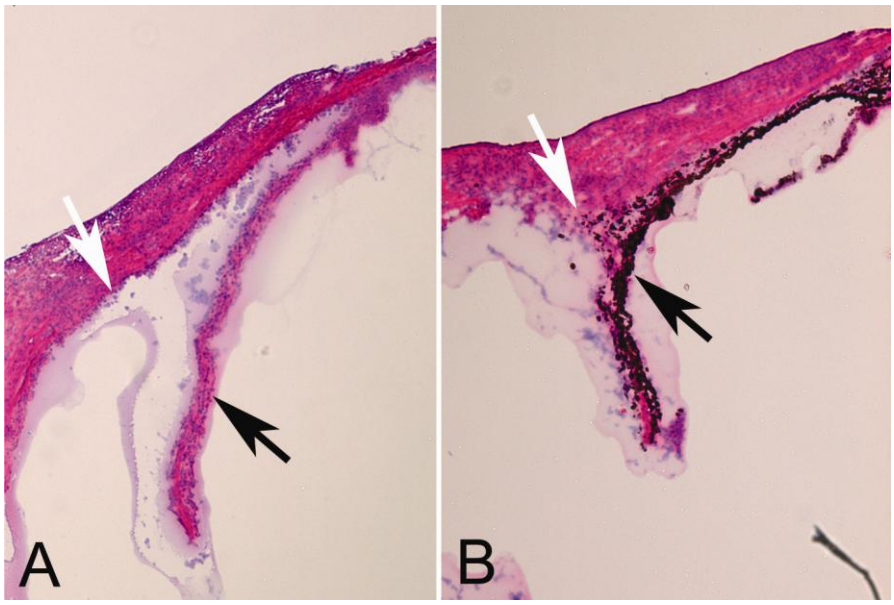


Figure 2.3 Anterior chamber drainage angle in eyes post- transplantation

Panel A shows part of a cross section of an A/J (albino) recipient eye which has received a corneal allograft. The anterior chamber drainage angle between the iris (black arrow) and the corneal endothelium (white arrow) is open with no synechia between the iris and the wound. Panel B shows part of a cross section of a C57BL/6(pigmented) recipient eye which has received a corneal allograft. The anterior chamber drainage angle between the iris (black arrow) and the corneal endothelium (white arrow) is closed due to synechia between the iris and the wound.

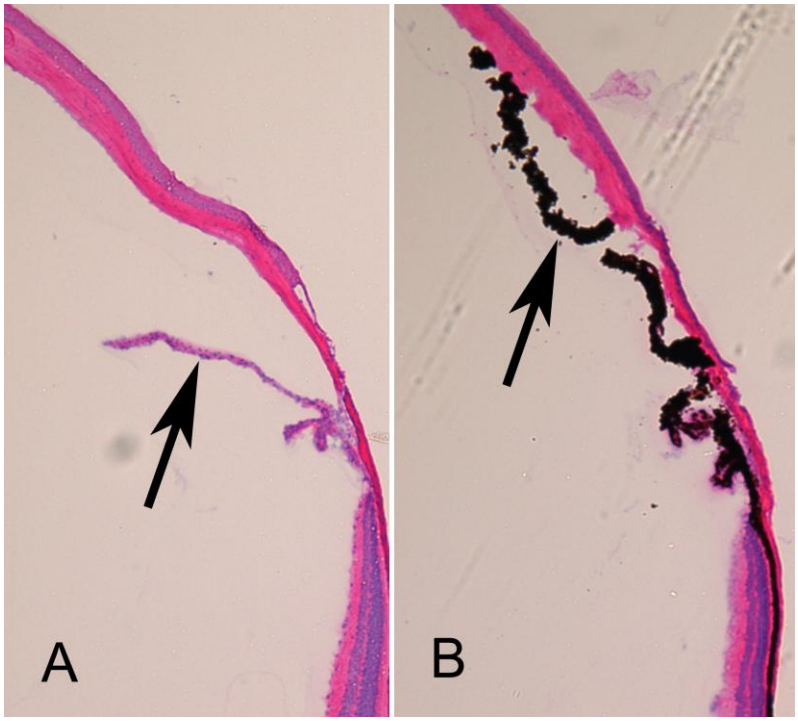


Figure 2.4 The iris in normal mouse eyes

Panel A shows part of a cross-section of a normal A/J (albino) eye stained with haematoxylin and eosin. The arrow indicates the iris which is relatively thin. Panel B shows part of a cross-section of a normal C57BL/6 (pigmented) eye stained with haematoxylin and eosin. The arrow indicates the iris which is relatively thick.

2.2.8. Pigmented Host: Grading system

1. Completely transparent cornea
2. Moderate corneal opacity, only pupil margin is visible
3. Complete corneal opacity, pupil not visible

The grading system in albino mice makes use of the clear visibility of iris vasculature by transillumination to allow subtle grading of corneal clarity. Iris detail is much less visible in pigmented mice and so the grading system in pigmented mice was modified accordingly. When grafting to pigmented recipients some investigators use this 1-3 grading system(Niederkorn et al., 2006b) whilst others persist with the more detailed scoring system used in albino mice. Corneal graft rejection was diagnosed when the corneal clarity score increased to 3 in a graft which was previously transparent following surgery (see Figure 2.5).

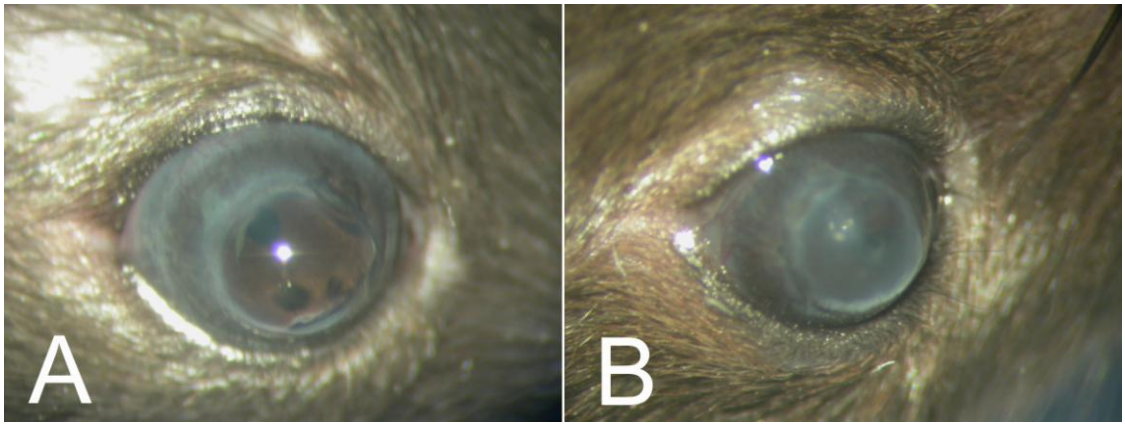


Figure 2.5 Diagnosis of corneal transplant rejection in pigmented mice

A. A transparent graft. Pupil and iris detail visible (grade 1). **B.** A rejected graft. Pupil no longer visible (grade 3).

2.2.9. Surgical success rate

When learning to perform orthotopic corneal grafts in albino mice, isografts were performed first so as to eliminate immune rejection as a cause of graft failure. i.e. failure of an isograft was due only to suboptimal surgical technique. Even in isografts initial surgical success rates were low, mostly due to cataract formation. With practice and due attention to surgical technique the success rate improved to 90% (Figure 2.6) and syngeneic grafts were found to become transparent at variable timepoints up to day 6.

Once surgical success rates were at 90% in albino mice transplantation was attempted in pigmented mice. Even using the modified technique, surgical success rates in pigmented eyes remained low and never improved above 40%. (Figure 2.6

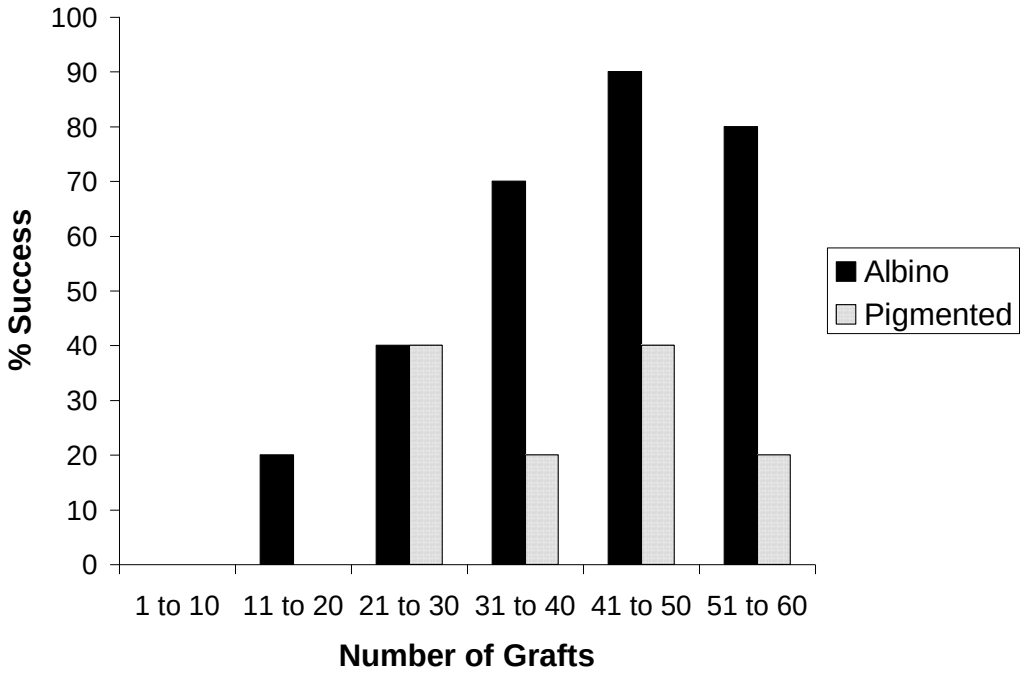


Figure 2.6 Success rate of corneal transplantation in mice

The bars represent the percentage surgical success rate within each group of 10 mice. For example in albino mice the success rate for grafts 1 to 10 was 0% and the success rate for grafts 11 to 20 was 20%.

2.3. EXPERIMENTAL ALLERGIC CONJUNCTIVITIS

Mice were sensitized to the allergen short ragweed pollen (SRW) over a 15 day period(Nakamura et al., 2003). This sensitization period is required for the generation of systemic Th2 responses and subsequent challenge with topical SRW results in severe allergic conjunctivitis. Mice were sensitized by intraperitoneal (i.p.) injection of short ragweed (SRW; Greer Laboratories, Inc, Lenoir, NC, USA) pollen 200 µg with 2 mg aluminium hydroxide as an adjuvant (Alum; Sigma. St. Louis, MO, USA) suspended in 0.4 ml phosphate-buffered saline (PBS; Invitrogen, Paisley, UK) on days 0, 7, and 14. When making the suspension for intraperitoneal injection, typically enough was made for 25 injections.

200µg x 25 = 5000µg = 5mg SRW

2mg x 25 = 50 mg Alum

400µl x 25 = 10000µl = 10ml PBS

The jar of short ragweed pollen was removed from the -20^{o}C refrigerator, its lid unscrewed slightly, and placed in a vacuum chamber with silica gel for 2 hours to prevent hydration while it heated to room temperature. SRW and Alum was measured by comparing volume with a given volume of PBS. Previous experiments by laboratory colleagues had established that :

The volume of 1mg SRW = 4µl, 5mg SRW = 20µl

The volume of 1mg Alum = 5µl, 50mg Alum= 250µl

The mixture was prepared in the fume hood with the air circulation turned off to prevent dispersion of the allergen. The base of the hood was covered in aluminium foil and paper towels soaked in 70% ethanol to absorb any spillage of allergen. Aliquots of 5mg SRW and 50mg Alum were made up as follows:

250µl of PBS was pipetted into an Eppendorf tube. With a spatula, an equal volume of Alum was carefully measured in to other sterile Eppendorf tubes.

20µl of PBS was pipetted into an Eppendorf tube. With a spatula, an equal volume of SRW was carefully measured in to other sterile Eppendorf tubes. The

tubes were then closed, wiped with 70% ethanol, sealed with laboratory film and stored at -20^{o}C until needed.

To make the suspension for 25 injections 8 ml of sterile PBS was pipetted into a sterile 10ml tube. 1ml of sterile PBS was added to an Eppendorf containing an aliquot of 50mg of Alum. This was mixed and transferred to the 8ml of sterile PBS in the 10 ml tube. 1ml of sterile PBS was added to an Eppendorf containing an aliquot of 5mg of SRW. This was mixed and transferred to the 10 ml tube. The suspension was vortexed for 30 minutes. For each injection 400µl of suspension was drawn into a 1 ml syringe. The mouse was held is such a way that the abdomen was exposed and the skin of the abdomen was reasonably taut. The suspension was injected intraperitoneally using a 25G needle.

The sensitization period also involved treatment with eyedrops (SRW pollen 500 µg with 25 µg Alum suspended in 5 µl PBS) to both eyes on days 8 and 15. When making the suspension for eyedrops for sensitisation, typically enough was made for 100 eyedrops.
500µg x 100 = 50000µg = 50mg SRW
25µg x 100 = 2500µg= 2.5mg Alum
5µl x 100= 500µl = 0.5ml PBS
Aliquots of 50mg SRW and 2.5mg Alum were made up in sterile Eppendorf tubes using the same method described above. To make the suspension 0.5ml sterile PBS was added to the tube containing the 2.5mg of Alum. This was mixed and transferred to the tube containing the 50mg SRW. The suspension was vortexed for 30 minutes.

To administer the eyedrops the mouse was held in one hand and 5µl of suspension was dropped onto the eye. The eyelids were then gently manipulated using a cotton bud to facilitate movement of the allergen into the conjunctival fornices.

The experimental challenge of 500 µg of SRW in 5 µl PBS was administered topically on day 27,.Eyedrops for challenge were made up in a similar way to those for sensitization the only difference being that no alum was added.
Using this protocol, allergic conjunctivitis was induced as evidenced by infiltration of the conjunctiva by large numbers of eosinophils at 48 hours post-challenge (Figure 2.7).

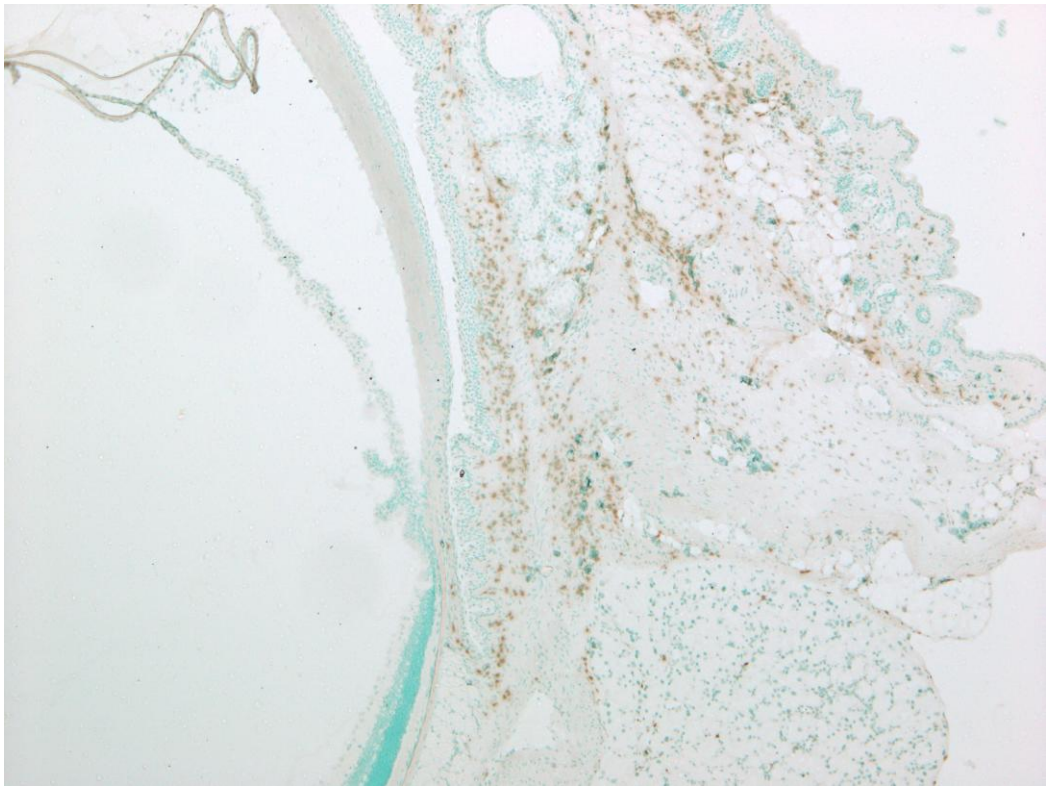


Figure 2.7 Allergic conjunctivitis in an A/J mouse

This figure shows part of a cross-section through the eyeball, conjunctiva and lid of an A/J mouse at 48 hours post-challenge with SRW. The section has undergone immunoperoxidase staining for Major Basic Protein (Eosinophils) and has been counterstained with Methyl Green (picture courtesy of Dr Masaharu Ohbayashi)

2.4. IMMUNOPEROXIDASE IMMUNOHISTOCHEMISTRY

2.4.1. Removal and embedding of mouse eyes

Once the desired timepoint/ endpoint was reached, mice were killed by CO_2 inhalation in a closed chamber and either the whole eye was enucleated or the eye was exenterated along with the conjunctiva and lids. To enucleate the eye, it was proptosed manually and a curved forceps was placed behind the globe. The retro bulbar tissues were gripped firmly and the eye enucleated, usually along with the optic nerve a pad of orbital fat and part of the conjunctiva.
Enucleated eyes were embedded in optimal cutting temperature compound (OCT compound; Sakura Finetek Europe BV, Zoeterwoude, The Netherlands) and oriented within the OCT compound so that the visual axis was parallel with the aluminium plate (Figure 2.8). The aluminium plate was then placed on a liquid nitrogen-cooled duralumin plate until the OCT compound had completely frozen. Specimens were stored at -70^{o}C.

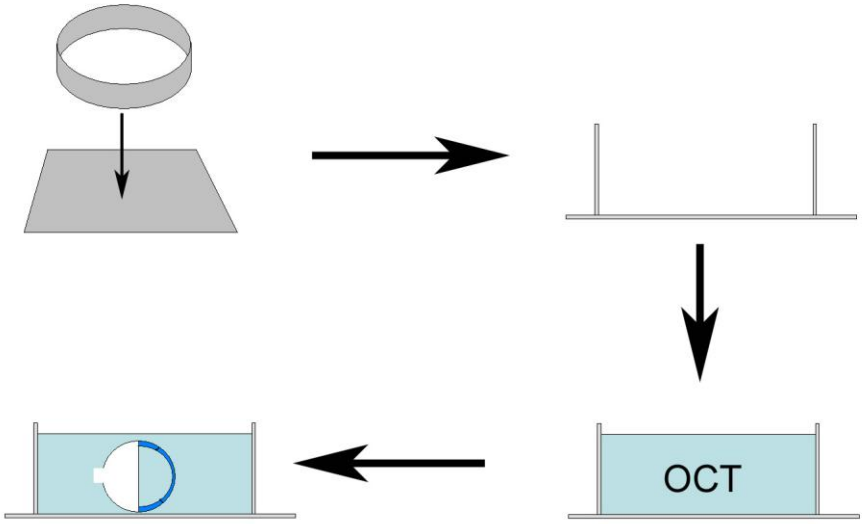


Figure 2.8 Embedding of the enucleated eye in OCT compound

An aluminium ring was placed on an aluminium plate. This creates a shallow cylindrical “mold” which is filled with OCT compound. The enucleated mouse eye is placed in the OCT compound and orientated so that the visual axis is parallel with the plate. Sectioning of the frozen cylinder provided an appropriate cross-sections of the cornea/ graft for analysis.

To exenterate the eye a stab was made with a sharp scissors through the cranium approximately 2 mm caudal to the lateral canthus. The scissors was then used to cut through the lids and periorbital bone in a pentagon shape as illustrated in Figure 2.9.

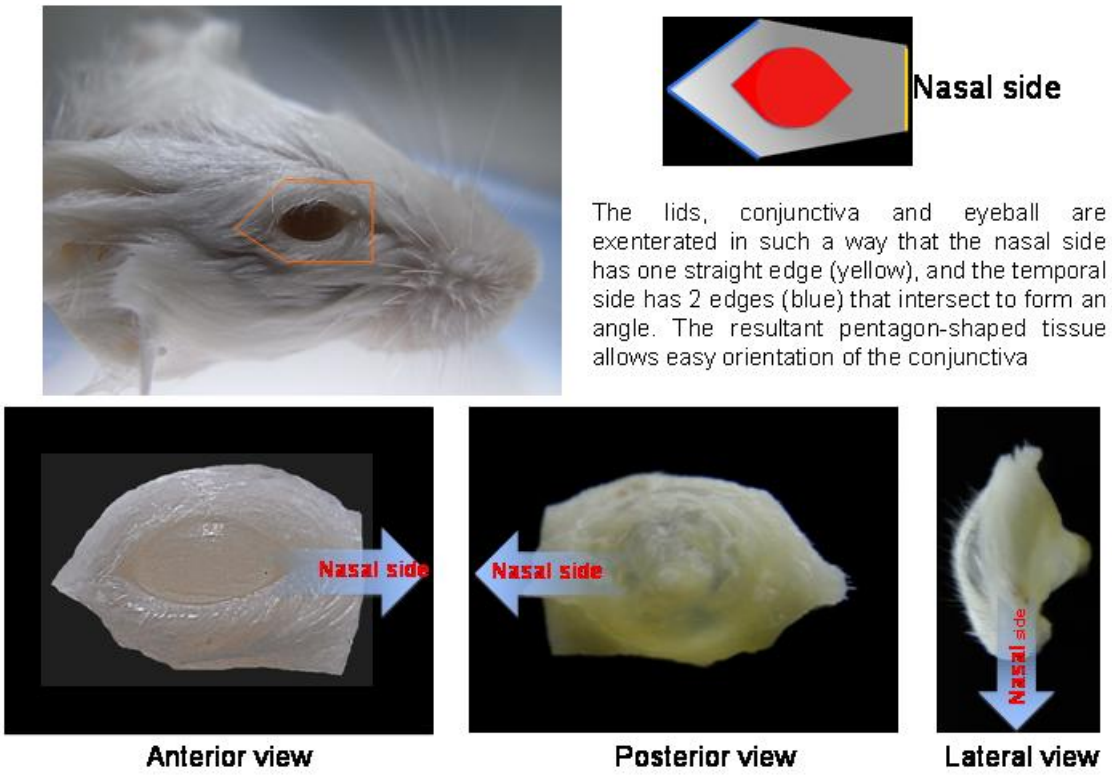


Figure 2.9 Exenteration of the mouse eye

This figure courtesy of Dr Masaharu Ohbayashi

The lids, conjunctiva and eyeball are exenterated in such a way that the nasal side has one straight edge (yellow), and the temporal side has 2 edges (blue) that intersect to form an angle (Figure 2.9). The resultant pentagon-shaped tissue allows easy orientation of the conjunctiva when cutting.

Following exenteration great care was taken to remove all bone from the specimen as this would have impaired the quality of frozen-sections. The specimen was embedded in OCT compound as illustrated in Figure 2.8 and frozen on a liquid nitrogen-cooled duralinium plate. Using this technique it was possible to preserve the normal morphology/ architecture of the conjunctiva and eyelids during sectioning (Figure 2.10).

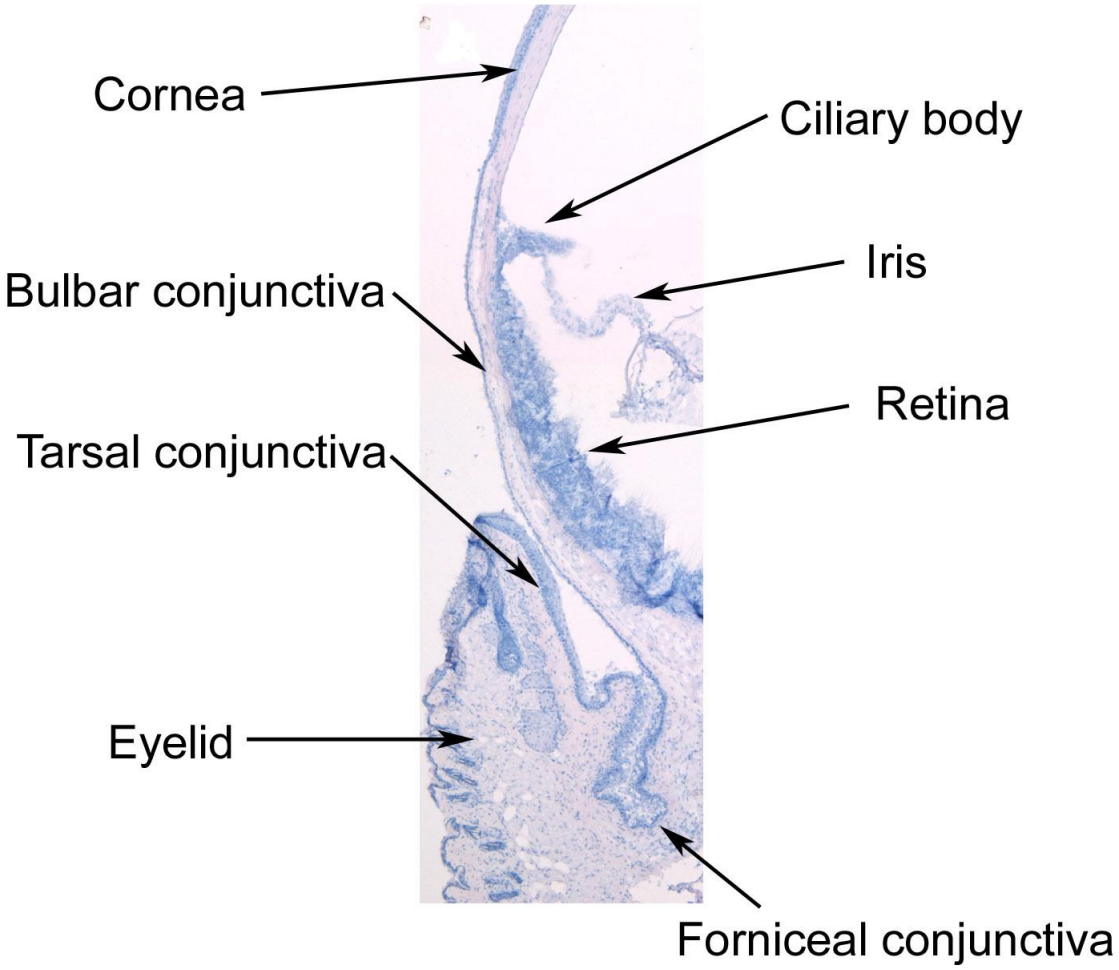


Figure 2.10 Histology of mouse eyelid and conjunctiva

2.4.2. Cutting and staining of sections

8µm thickness cryostat sections were cut and allowed to dry in air for 1 hour and circled with a "DAKO" pen (Dako, Ely,UK). Sections were fixed in a mixture of 30% methanol and 70% acetone (VWR International, Lutterworth, UK) at 4^{o}C for 5 minutes and then washes in PBS three times for 5 minutes each time. Sections were then incubated with 200µl 5% skimmed milk in a moist chamber at room temperature for 30 minutes to block non-specific protein-binding sites. During incubation primary antibody was prepared by diluting it appropriately in 1% Bovine Serum Albumin (BSA; Sigma-Aldrich, Poole, United Kingdom) in PBS (100ml PBS + 1g BSA + 0.01g sodium azide [Sigma-Aldrich, Poole, United Kingdom]).

The following primary rat anti-mouse antibodies were used:

Clone	Target	Cellular distribution/ function	Isotype	Dilution	Company
RM4-5	Mouse CD4	Found on T helper and Regulatory T cells. It is a co-receptor for the T cell receptor with specificity for MHC class II.	IgG2A	1:100	BD (Oxford, UK)
YTS105.18	Mouse CD8	Found on T cytotoxic cells. It is a co-receptor for the T cell receptor with specificity for MHC class I.	IgG2A	1:100	Serotec (Kidlington, UK)
CI:A3-1	Mouse F4/80	Found on the surface of mouse macrophages of myeloid origin.	IgG2b	1:300	Serotec
	Mouse MBP	Found in granules of eosinophils. It induces histamine release and is directly toxic to helminths and mammalian cells	IgG1	1:400	Dr. Lee (Mayo Clinic)*
Isotype control			IgG2A	1:100	Serotec
Isotype control			IgG2b	1:100	Serotec
Isotype control			IgG1	1:400	Serotec

Table 2.1 Antibodies used in peroxidase immunohistochemistry

* This antibody was kindly provided as a gift by Dr. Lee

The following parameters were optimised by practicing staining on positive control specimens (mouse spleen) and failed corneal allografts (Figure 2.6):

- Dilution of primary antibody (1:50 – 1:400)
- Length of incubation of primary antibody (1hour v overnight)
- Temperature of incubation of primary antibody (room temperature v 4^{o}C)

Blocking solution was shaken off and 200µl of primary antibody were added and incubated overnight at 4 ^{o}C in a moist chamber. Sections were then washed three times in PBS and placed in a solution of 145 ml methanol and 5 ml 30% hydrogen peroxide (VWR International, Lutterworth, UK) (1%H_20_2 in 100% methanol) for 20 minutes at room temperature to quench endogenous peroxidase acivity. Sections were washed 3 times in PBS and then incubated with 200µl universal immunoperoxidase polymer for mouse tissue sections (Histofine® Simple Stain Mouse Max PO; Nichirei Biosciences, Tokyo, Japan) for 1 hour in a moist chamber at room temperature. Sections were then washed 3 times in PBS. 8 tablets of Tris buffer (Sigma, Poole, UK) were dissolved in 120 ml of distilled water. 20mg of diaminobenzidine (VWR International Ltd, Lutterworth, UK) was added to this solution and mixed for 10 minutes. 200µl of H_20_2 was added to the solution. Slides were placed in the dH_20/Tris/DAB/H_20_2 solution for approximately 2 minutes. Strength of the reaction was gauged by observing a positive control slide under the microscope for a few seconds. After approximately 2 minutes the DAB reaction was stopped by washing slides in PBS and then in distilled water. Sections were counterstained by dipping in haematoxylin (Fluka BioChemica, Buchs, Switzerland) and washing in distilled water until clear. 4 containers of ethanol (VWR International Ltd, Lutterworth, UK) for differentiation were prepared: 70% (30 ml dH_2O + 70 ml Ethanol)*;* 95% (5ml dH_2O + 95 ml Ethanol)*;* 100% *;* 100%. Slides were dipped in each solution 10 times and left submerged in the final container for 2-5 minutes. Slides were dipped 10 times in each of 3 xylene (VWR International Ltd, Lutterworth, UK) solutions and left in the last container for 2-5 minutes. Slides were mounted with glass covers and DPX mounting medium (VWR International Ltd, Lutterworth, UK).

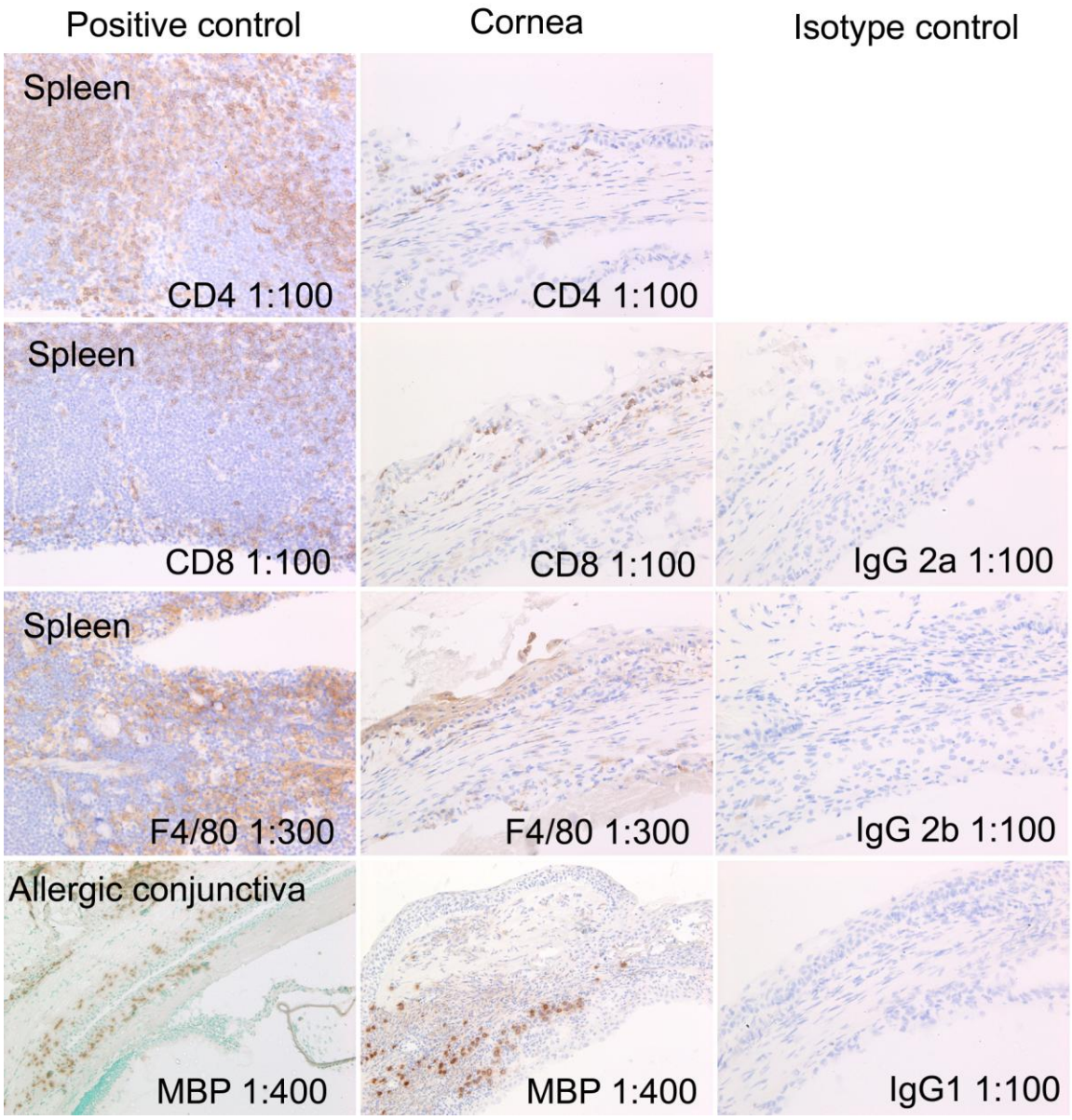


Figure 2.11 Peroxidase immunohistochemistry of corneal allografts

Concentrations of anti-CD4, anti-CD8 and anti-F4/80 antibodies were optimised using mouse spleen as a positive control and using failed corneal allografts. A concentration of 1:400 for the anti-MBP antibody was recommended by a colleague who had experience using this antibody on mouse conjunctiva. On examining rejected corneal allografts in allergic graft recipients it became apparent that this concentration was suitable for corneal staining also.

2.5. FLUORESCENT IMMUNOHISTOCHEMISTRY

2.5.1. Introduction

8µm thickness cryostat sections were cut and allowed to dry in air for 1 hour and circled with a "DAKO" pen. Sections were fixed in a mixture of 30% methanol and 70% acetone at 4^{o}C for 5 minutes and then washes in PBS three times for 5 minutes each time. Sections were stained first with a green fluorochrome, either by direct or indirect immunohistochemistry and then with PE-conjugated anti-CD11b.

2.5.2. Direct staining with FITC-labelled antibody

For direct staining sections were blocked with a 1:100 solution (diluted in 1% BSA in PBS) of 5µg/ml anti mouse CD16/CD32 ("Fc blocker"; BD, Oxford, UK) at room temperature for 30 minutes in a moist chamber to block endogenous Fc binding. During incubation primary antibody was prepared by diluting it appropriately in 1% BSA in PBS. The primary antibody which was fluorescence-labelled was spun down before use (10,000 rpm for 10 minutes at 4 ^{o}C). Antibodies used for direct fluorescent staining were:

Clone	Target	Isotype	Fluorochrome	Conc	Company
CI:A3-1	F4/80	IgG2b	FITC	1:100	Serotec
Isotype control		IgG2b	FITC	1:100	BD

Table 2.2 Antibodies used for direct staining in fluorescent immunohistochemistry

Blocking solution was shaken off and 200µl of primary antibody were added and incubated for 2 hours at room temperature in the dark in a moist chamber. Sections were then washed three times in PBS, this and all subsequent steps being performed in darkness (using aluminium foil to protect from light where necessary).

2.5.3. Indirect staining with Alexa Fluor 488 -labelled antibody

For indirect staining sections were blocked first with 5% skimmed milk for 30 minutes at room temperature in a moist chamber and then with 10% normal donkey serum (Stratech Scientific, Newmarket, UK) in PBS for 30 minutes at room temperature in a moist chamber. During incubation primary antibody was prepared by diluting it appropriately in 1% BSA in PBS. Antibodies used for indirect fluorescent staining were:

Clone	Target	Cellular distribution/ Function	Isotype	Dilution	Company
223322	LYVE-1	A cell surface hyaluronan receptor found on lymphatic endothelial cells.	IgG2a	1:400	R&D systems (Abingdon, UK)
NIMP-R14	Gr-1	This antigen is found on the cell surface of a variety of myeloid-derived cells. Often used as a marker of neutrophils it is seen on monocytes during differentaition and also found on myeloid suppressor cells.	IgG2b	1:400	AbCam (Cambridge, UK)
Isotype control			IgG2a	1:400	Serotec
Isotype control			IgG2b	1:400	Serotec

Table 2.3 Antibodies used for indirect staining in fluorescent immunohistochemistry

Blocking solution was shaken off and 200µl of primary antibody were added and incubated for 2 hours at room temperature in a moist chamber. Sections were then washed three times in PBS. Secondary (fluorescence-labelled) antibody

was prepared by diluting it appropriately in 1% BSA in PBS and spinning it down before use. Secondary antibody used was Alexa 488-conjugated Donkey anti-Rat IgG (MolecularProbes, Invitrogen, Paisley, UK; clone A-21208) used at dilution of 1:1000. 200µl of secondary antibody were added and incubated for 1 hour at room temperature in the dark in a moist chamber. Sections were then washed three times in PBS, this and all subsequent steps being performed in darkness (using aluminium foil to protect from light where necessary).

2.5.4. Direct staining with PE-labelled anti-CD11b

Primary antibody was prepared by diluting it appropriately in 1% BSA in PBS and spinning down before use (10,000 rpm for 10 minutes at 4 ^{o}C). Antibody used for direct fluorescent staining was:
Rat anti Mouse CD11b-PE (BD) (Control PE- Rat IgG2b)

Clone	Target	Isotype	Fluorochrome	Conc	Company
M1/70	CD11b	IgG2b	PE	1:100	BD
Isotype control		IgG2b	PE	1:100	BD

Table 2.4 Antibodies used for direct staining in fluorescent immunohistochemistry

200µl of primary antibody were added and incubated for 1 hour at room temperature in the dark in a moist chamber. Slides were washed in distilled water and mounted with glass cover slips and fluorescence gel mounting medium.
Cellular staining with PE-conjugated anti-CD11b under these conditions worked well but had one major disadvantage in that the fluorescence faded extremely quickly during imaging which meant the sections could be imaged only once.

Staining results of fluorescent immunohistochemistry using the methods described here are shown in Figure 2.12.

	Blocking		First Colour						Second Colour		
Fc	**Sk Milk**	**nds**	**1°**	**x**	**inc**	**2°**	**x**	**incubation**	**Anti-**	**x**	**incubation**
	+	+	LYVE 1	40 0	2 hours @ RT	Alexa 488	1000	1 hour @ RT	CD11b	10 0	1 hour @ RT
+			F4-80	10 0	2 hours @ RT	-	-	-	CD11b	10 0	1 hour @ RT
	+	+	Gr1	40 0	2 hours @ RT	Alexa 488	1000	1 hour @ RT	CD11b	10 0	1 hour @ RT

Table 2.5 Summary of the protocol for fluorescent immunohistochemistry

Fc= anti-CD16/anti-CD32 antibody (“Fc blocker”)
NDS= Normal Donkey serum
RT= Room Temperature

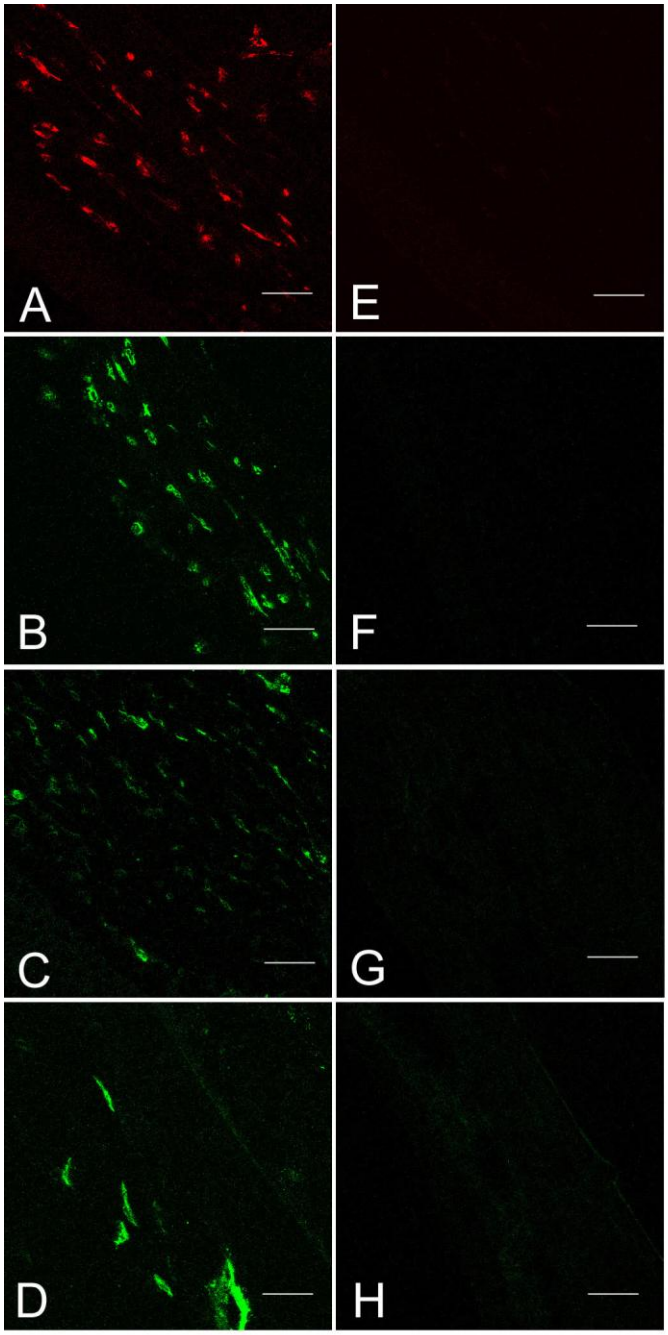


Figure 2.12 Fluorescent immunohistochemistry of mouse host cornea 2 days after corneal transplantation

Cells infiltrating the host cornea were imaged on a Zeiss LSM510 confocal microscope (x400 magnification). Cells stain positively for CD11b (A), Gr-1 (B) and F4/80 (C) but are not stained by the corresponding isotype control antibodies (E-G). Anti-LYVE-1 antibody stained structures at the limbus (D) but these did not stain with the isotype control antibody (H). White scalebar = 50μm

2.6. CORNEAL WHOLEMOUNT

Cross-sections of mouse corneal grafts were used to stain and count cells entering the host cornea following corneal transplantation. To study the ingrowth of new lymphatic vessels following transplantation I considered using cross-sections from the same eyes as were used to study cellular infiltrates. Lymphatic vessels in these sections can be stained with antibody against LYVE-1, a marker for lymphatic endothelium (Figure 2.13). An advantage of this method would be that fewer animals would be needed as sections from the same specimen eyes as were used for analysis of cellular infiltration could be used but this method would have several disadvantages. Firstly, on cross section lymphatics are measurable in one dimension only. Secondly, a section may "hit" or "miss" the lymphatic vessels which grow in an approximate radial fashion and so may not accurately reflect the extent of lymphatic ingrowth.

After consideration, the final decision was to repeat the experiment and perform wholemount staining of the corneas with LYVE-1 as i) this would give a more comprehensive measure of corneal lymphangiogenesis and ii) this was the method used by other investigators.

Whole eyes were excised at days 2 and 6 post-transplantation. The sclera was punctured and eyes were fixed in acetone for 1 hour at room temperature. The cornea was excised at the limbus and any remaining conjunctiva was removed. With a blade, four slits were made in the peripheral cornea at 90^{o} to each other to give the cornea a cruciform shape and facilitate flat-mounting. Corneas were washed in PBS 6 times for 5 minutes each time. They were blocked with 2% BSA in PBS for 1 hour at room temperature and with 10% normal donkey serum for 1 hour at room temperature. During incubation primary antibody was prepared by diluting it appropriately in 1% BSA in PBS. Antibody used for indirect fluorescent staining was:

Anti-LYVE-1 (R&D) CLONE: 223322 dilution 1:1000

Corneas were incubated overnight at room temperature in a six-well plate containing primary antibody. After 6 washes in PBS they were incubated for 1 hour at room temperature in darkness with Alexa488-conjugated donkey anti-rat antibody followed by washing and mounting with fluorescence mounting gel medium and glass covers.

Wholemounts were imaged by fluorescent microscopy (Olympus). An image of each quadrant was captured with original magnification of x100. Radial ingrowth of LYVE-1^+ vessels from the limbus was measured in each quadrant by a masked observer using image analysis software (Soft Imaging System GnbH, Munster, Germany). First the system was calibrated by photographing a 1mm scale at the same magnification (x100). Using a freehand drawing tool on the software a line was traced manually over each lymphatic vessel and its branches. The software automatically calculates the length of each line and these values were recorded and the combined total length of lymphatic vessels in each quadrant was calculated. The totals for each quadrant were added to give a measure of lymphatic ingrowth for each cornea. At least 4 corneas were analysed per group per timepoint. Mean values were calculated.

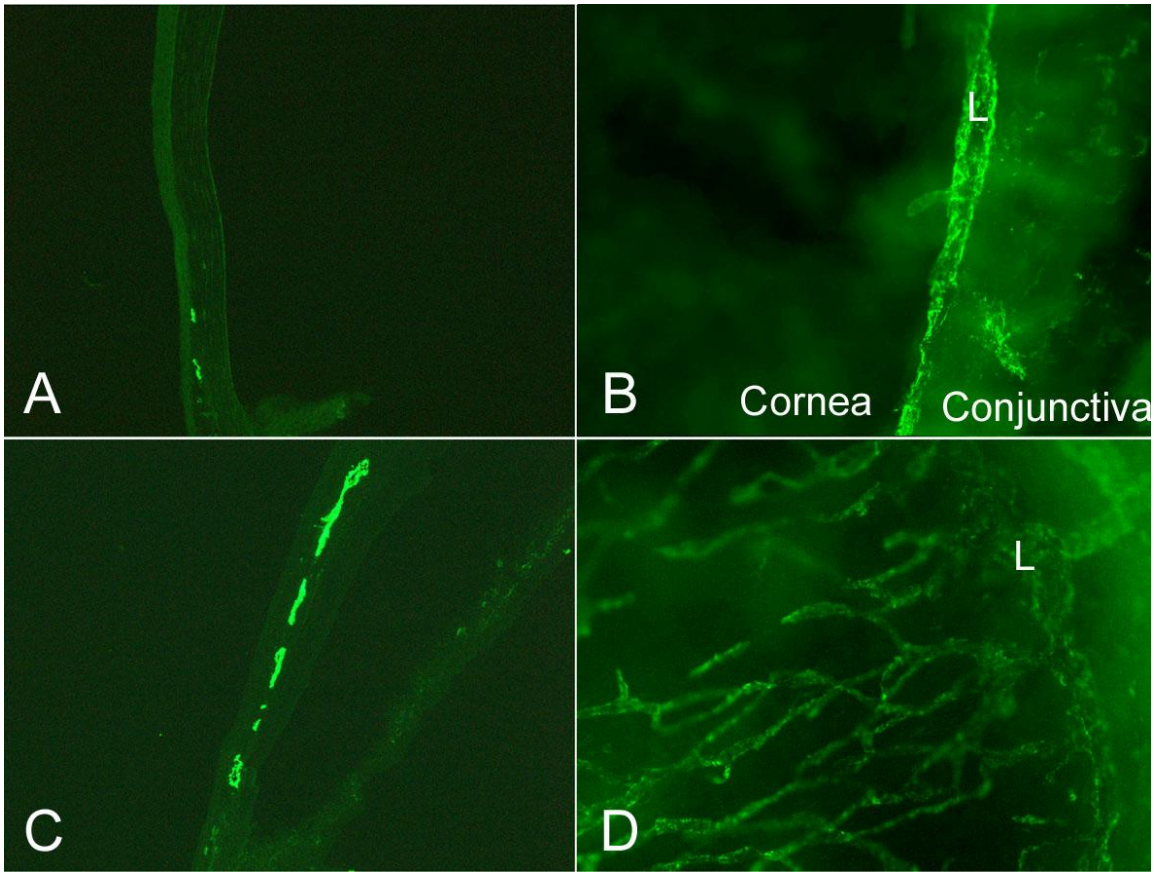


Figure 2.13 LYVE-1 expression in cornea after transplantation

There is relatively little expression of LYVE-1, a marker for lymphatic endothelium, in normal mouse cornea. This is demonstrated in panels A and B which show a details of a frozen section (original magnification x100) and a corneal wholmount (original magnification x100) respectively. In panel B there is extensive LYVE-1 expression on vessels in the conjunctiva and the limbus (L) and there are relatively few LYVE-1 vessels extending into the peripheral cornea. 6 days after corneal allotransplantation subepithelial linear ingrowth of LYVE-1^{+} structures is seen both on frozen section (C) and corneal wholemount (D).

2.7. FLOW CYTOMETRY

2.7.1. General considerations

Flow cytometry is a technique used to study characteristics of particles suspended in a liquid. In biology the particles most often analysed are cells and the specific cells of most interest to immunologists are leukocytes. Cell types may be distinguished on the basis of physical charactersistics such as size (measured by forward scatter [FSC]) or granularity (measured by side scatter [SSC]). Fluorochrome-conjugated antibodies may be used to measure the expression of specific cell surface (or intracellular) proteins on cells. The outputs of such analsyses are typically histograms which describe the expression of a single protein/antigen and scatter plots which correlate the expression of two proteins/antigens.

2.7.2. Preparation of peripheral blood for flow cytometry

First, using peripheral blood from a normal volunteer, staining conditions and flow cytometry parameters were optimised for the antibodies listed in Table 2.3. This section describes the technique used to stain cells with these antibodies. The rationale for my choice of antibodies to use for the analysis is explained in the introduction to Chapter 5.

Clone	CD	Isotype	Fluorochrome	Activity	Company
SK3	CD4	IgG_1	FITC	Helper T Cell	BD
SK1	CD8	IgG_1	PE	Cytotoxic T Cell	BD
mØP9	CD14	IgG_{2b}	APC	Monocyte/ Macrophage	BD
2D1	CD45	IgG_1	PerCP	Pan-leukocyte	BD
Isotype control		IgG_1	FITC	Isotype control	BD
Isotype control		IgG_1	PE	Isotype control	BD
Isotype control		IgG_{2b}	APC	Isotype control	eBioscience(Hatfield, UK)
Isotype control		IgG_1	PerCP	Isotype control	BD

Table 2.6 Antibodies used in flow cytometry of blood and aqueous

Two 100µl samples of anticoagulated whole peripheral blood were transferred to EDTA-coated microtubes. One tube was stained with 5µl each of fluorochrome-labelled anti-CD45, anti-CD4, anti-CD8 and anti-CD14 monoclonal antibodies (Table 2; all antibodies from BD except the APC-conjugated IgG_{2b} isotype control (eBioscience SanDiego, USA)). The other was stained with fluorochrome-labelled isotype-matched control antibodies. Cells were incubated with antibodies for 40 minutes in darkness at room temperature.

Then each sample was mixed with 1 ml of lysis buffer (BD), allowed to stand for 10 minutes at room temperature and then centrifuged at 300g for 5 minutes at 4°C. The cell-free supernatant was then discarded and the cells washed once with PBS, resuspended in PBS and analysed immediately by 4-colour flow cytometry.

2.7.3. Acquisition of data

Events were acquired using a Partec flow cytometry machine (Partec, Munster, Germany). The flow cytometry settings were optimised by repeating flow cytometry on peripheral blood from a healthy volunteer. Once optimised this technique and was used to analyse peripheral blood from patients with and without corneal allograft endothelial rejection in the experiments described in Chapter 6. Typically 6000 events were recorded and the typical settings used on the flow cytometer when acquiring data for peripheral blood were:

Speed: 5.0						
Enable	Parameter		Gain	Log	L-L	U-L
	FSC	-	141.5	log3	50.0	999.9
	SSC	-	135.5	log3	10.0	999.9
	FL1	-	420.0	log4	10.0	999.9
	FL2	-	429.0	log4	10.0	999.9
	FL3	-	472.0	log4	10.0	999.9
	FL4	-	396.0	log4	10.0	999.9

Table 2.7 Typical flow cytometry parameters for acquisition of data from peripheral blood

2.7.4. Analysis of data

Data were analysed using Flowmax analysis software (Partec). In the first instance events were analysed using a scatter plot of forward scatter(FSC) v side scatter (SSC) and histograms displaying fluorescent activity collected in fluorescence 1 (FL1, green channel), fluorescence 2 (FL2, orange-red channel), fluorescence 3 (FL3, red channel) and fluorescence 4 (FL4, deep red channel) respectively.

Distinct clusters of events could be seen on the scatter plots of peripheral blood (Figure 2.14; A-C). The settings on the flow cytometer were adjusted so that the histogram peaks in FL1, 2, 3 and 4 for unstained blood lay at the left of the plot (Figure 2.14; A). Exposure of the blood to PerCP-linked anti-CD45 increased the fluorescence of the sample in FL3 and moved the histogram peak to the right

(Figure 2.14; C) showing that these cells were $CD45^+$. A “twin peak” of CD45 staining was typically seen reflecting the fact that lymphoytes express more CD45 than granulocytes. Exposure of the blood to isotype controls had no effect on the position of the histogram peak (Figure 2.14; B).

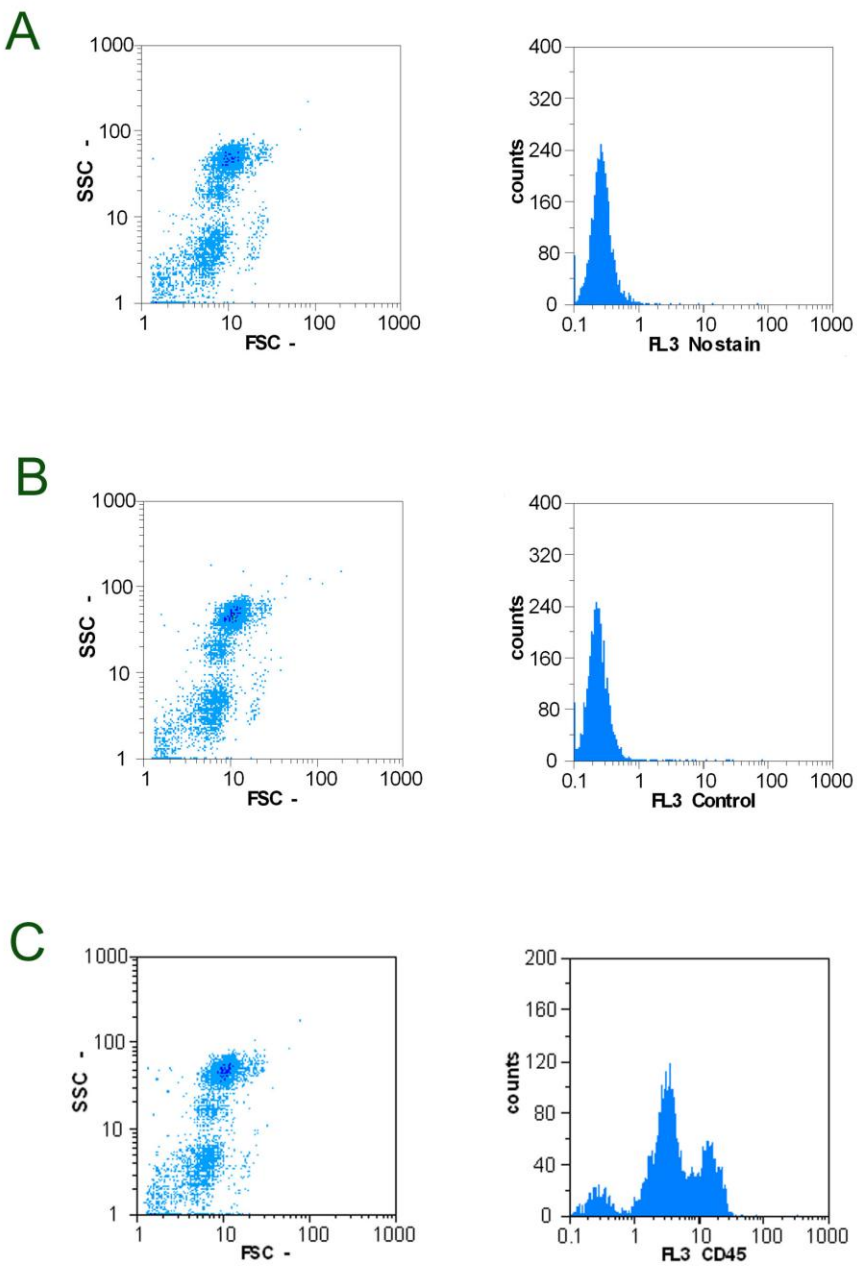


Figure 2.14 Staining with PerCP-labelled anti-CD45 of peripheral blood from a normal volunteer

Distinct clusters of events could be seen on the scatter plots of peripheral blood (A-C). The settings on the flow cytometer were adjusted so that the histogram peak for unstained blood lay at the left of the plot (A). Exposure of the blood to PerCP-linked anti-CD45 increased the fluorescence of the sample and moved the histogram peak to the right (C) showing that these cells were $CD45^{+}$. Exposure of the blood to isotype controls had no effect on the position of the histogram peak (B).

When analysing events in flow cytometry a specific subset of events is often of particular interest and the way in which these events are isolated is known as “gating”. For my analyses I was interested in white blood cells only. The physical characterstics of individual leukocyte sub-populations on the FSC/SSC plot have been well-described(Calvelli et al., 1993) and investigators typically use this plot to isolate or “gate” the cells of particular interest.

To ensure that only leukocytes were analysed a gate could be drawn manually around the leukocyte clusters on the FSC/SSC plot. An alternative strategy would be to use staining with the CD45 (pan-leukocyte) antibody to define which events were leukocytes. Gating on the $CD45^{+}$ events confirmed that these events were the same as those within the known leukocyte clusters on the scatter plot (Figure 2.15 A&B). Therefore either technique could be used to gate on leukocytes. For experiments I decided to use a gate drawn manually around the leukocyte cluster as defined by their physical properties on the FSC/SSC scatter plot (Figure 2.15 C) as this is the strategy used by most investigators.

The results for staining with antibodies against CD4, CD8 and CD14 are shown in Figure 2.16. A second (smaller) peak was seen to the right of the main peaks in FL1, 2 and 4 respectively. This shows that a certain proportion of the events in the sample were positive for CD4 (Figure 2.16; A), CD8 (Figure 2.16; B) and CD14 (Figure 2.16; C) respectively. No second peak was seen in analyses of samples exposed to isotype-matched controls. (Figure 2.16; D,E,F).

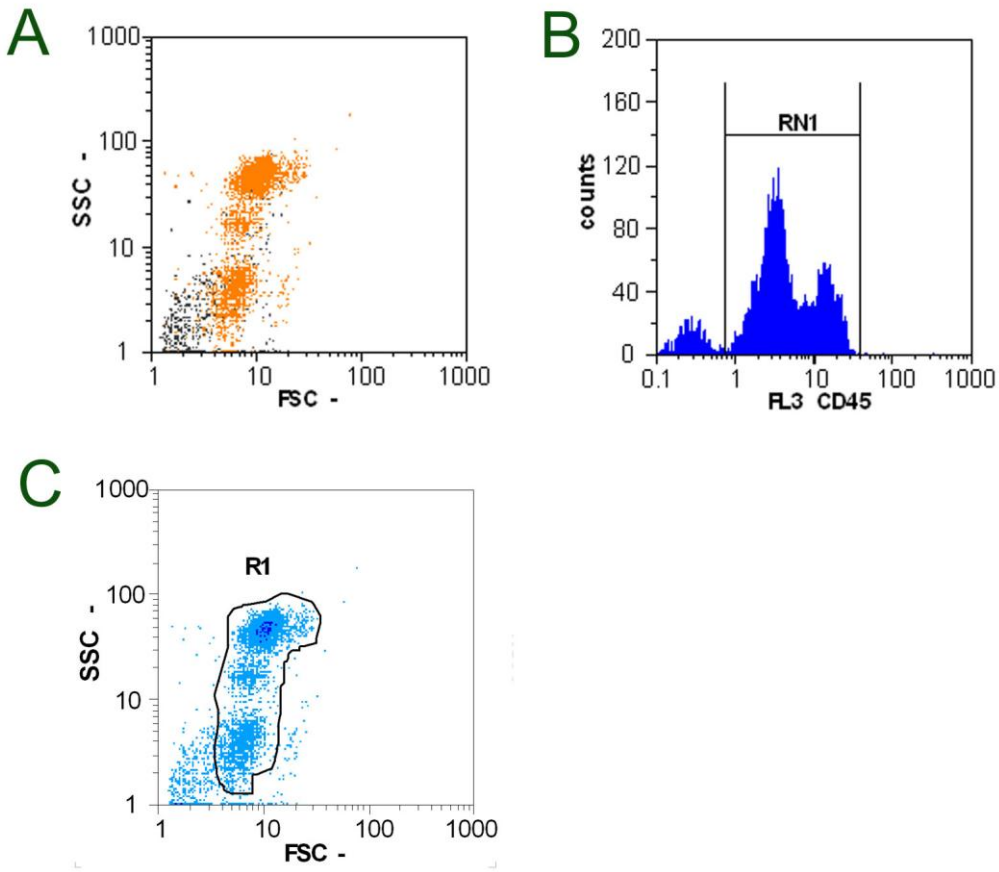


Figure 2.15 Gating on leukocytes in peripheral blood

A region gate (RN1) was selected on the histogram depicting CD45 staining of peripheral blood cells to select $CD45^+$ events (B). On the scatter plot events that fall within this gate are shown in red while other events are black (A). Red (i.e. $CD45^+$) events correspond with the known leukocyte clusters on the scatter plot (C).

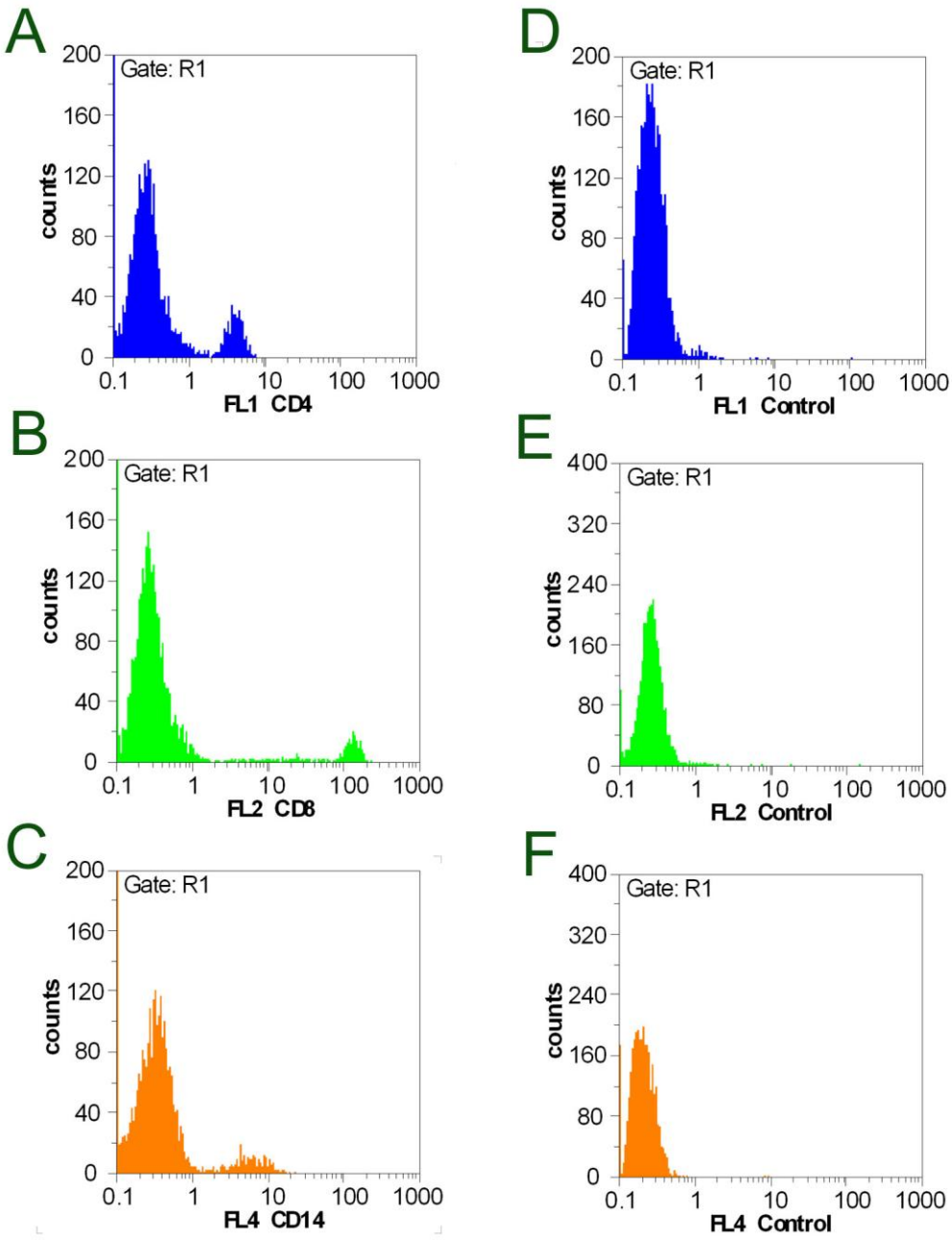


Figure 2.16 Optimising anti-CD4, anti-CD8 and anti-CD14 antibodies

A proportion of the $CD45^{+}$ events were also positive for CD4 (A), CD8 (B) and CD14 (C) respectively as evidenced by second peak on each histogram. No second peak was seen on histograms of peripheral blood stained with isotype controls (D-F).

2.8. CYTOMETRIC BEAD ARRAY

2.8.1. Aqueous and blood samples

Cytometric bead array sets (Becton Dickinson) were used to detect the following proteins:

Cytokines	Chemokines
IL-2	MCP-1
IL-4	RANTES
IL-6	MIP-1α
TNF-α	Eotaxin
IFN-γ	IP-10

Table 2.8 Proteins measured by cytometric bead array

2.8.2. Preparation of CBA Human Soluble Protein Flex Set Standards

A lyophilized standard sphere for each set was placed in a 10 ml tube. The standards were reconstituted with 4 ml of Assay Diluent from the Master Buffer kit (BD) and allowed to equilibrate for 15 minutes at room temperature. 10ml tubes were labeled and arranged in the following order: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256. 500 µL of Assay Diluent was pipetted to each tube. A dilution was performed by transferring 500 µL from the Top Standard to the 1:2 dilution tube and mixing thoroughly . Serial dilutions were continued by transferring 500 µL from the 1:2 tube to the 1:4 tube and so on to the 1:256 tube and mixing thoroughly. One tube containing 500 µL of Assay Diluent alone was prepared to serve as the 0 pg/mL negative control.

The approximate concentration (pg/mL) of each BD™ CBA Human Soluble Protein Flex Set Standard in each dilution tube is shown in Table 2.9.

BD CBA Human Soluble Protein Flex Set Standard	Top Standard	1:2 Dilution Tube	1:4 Dilution Tube	1:8 Dilution Tube	1:16 Dilution Tube	1:32 Dilution Tube	1:64 Dilution Tube	1:128 Dilution Tube	1:256 Dilution Tube
Protein (pg/mL)	2500	1250	625	312.5	156	80	40	20	10

Table 2.9 Cytometric Bead array human soluble protein flex set standard concentrations after dilution

This table was taken from the Becton Dickinson cytometric bead array human soluble protein master buffer kit instruction manual.

2.8.3. Preparation of CBA Capture Beads

Samples	Number of tests
Standard dilutions	10
Aqueous (rejection)	11
Aqueous (control)	8
Serum (rejection)	11
Serum (control)	8
Total	**48**

Table 2.10 Number of tests in cytometric bead array

Enough capture beads were prepared for 55 tests. Capture beads are supplied in a concentrated form so that 1.0 µL = 1 test. Each capture bead stock vial was vortexed to resuspend the beads.

Total volume of concentrated beads required for 55 tests = 10x55x1 µL= 550 µL

Total volume of diluted beads required for 55 tests = 55 x 50 = 2750 µL

Volume of capture bead diluent requried for 55 tests = 2200 µL

55 µL of each capture bead was added to an Eppendorf tube. 0.5 ml of wash buffer was added and the tube centrifuged at 200 g x 5 minutes. The supernatant was carefully aspirated and discarded. The beads were resuspended in Capture bead diluent, transferred to a 10 ml tube and further diluted with Capture bead diluent to a final volume of 2750 µL.

2.8.4. Preparation of PE Detection Reagents

PE Detection Reagents were prepared for 55 tests. PE Detection Reagents are supplied in a concentrated form so that 1.0 µL = 1 test. Care was taken to protect the PE Detection Reagents from exposure to direct light at all times.
Total volume of concentrated PE Detection Reagents required for 55 tests = 10x55x1 µL= 550 µL
Total volume of diluted PE Detection Reagents required for 55 tests = 55 x 50 = 2750 µL
Volume of PE Detection Reagent diluent requried for 55 tests = 2200 µL
2200 mL of PE Detection Reagent diluent was pipetted to a 10 mL tube wrapped in aluminium foil. 55 µL of each PE Detection Reagent was added to the tube which was stored at 4^{o}C until use.

2.8.5. BD CBA Human Soluble Protein Flex Set Assay Procedure

48 Eppendorf tubes were prepared for assay. The mixed capture beads were vortexed for 5 seconds and 50 µL added to each assay tube. 50 µL of standard dilutions were added to the first 10 tubes. 50 µL of the 38 samples to be tested were added to the other tubes. The assay tubes were mixed gently and incubated for 1 hour at RT. 50 µL of the Mixed PE Detection Reagent were then added to each assay tube. Assay tubes were mixed gently and incubated for 2 hours at RT.

After incubation 1 mL of Wash Buffer was added to each tube and tubes were centrifuged at 200 x g for 5 minutes at RT. The supernatants were then carefully aspirated and discarded. 300 µL of Wash Buffer was added to each assay tube and tubes were vortexed briefly to resuspend the beads.

2.8.6. Acquisition and analysis

Assay tubes were analysed immediately by two-colour flow cytometry (FACScan; BD). Analysis was performed using CBA software (Cellquest, FCAPArray; BD) which creates standard curves using data from the serial dilutions of known concentrations of recombinant human cytokines and chemokines (Figure 2.17) and uses these curves to convert fluorescence units of individual samples to cytokine and chemokine concentrations (pg/ml).

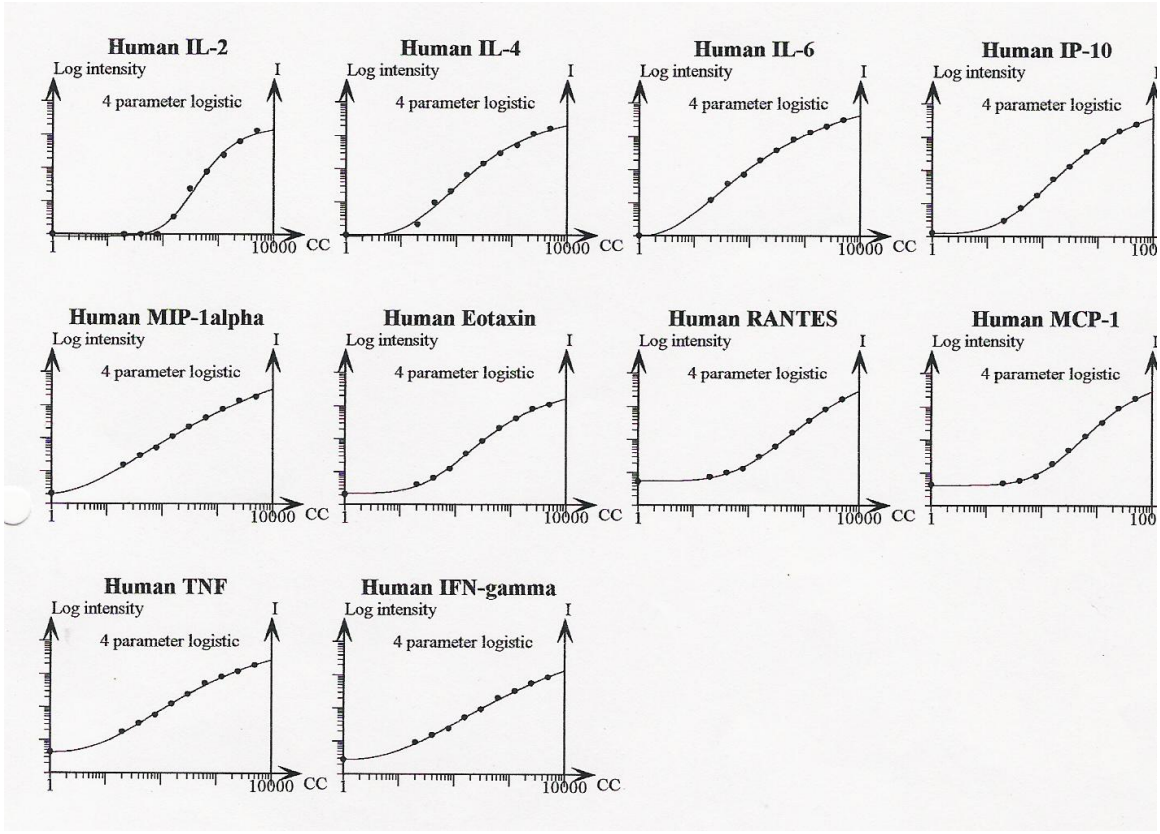


Figure 2.17 Standard curves constructed using data from the serial dilutions of known concentrations of recombinant human cytokines and chemokines

2.9. RNA EXTRACTION

2.9.1. General considerations

Earlier experiments by laboratory colleagues indicated that at least 20µg of RNA would be necessary for a ribonuclease protection assay. To achieve this it had been found that at least 3 whole eyes would be required per sample.

2.9.2. Extraction of RNA

Whole eyes were removed at day 12 post keratoplasty. 3 eyes were homogenised in 1 ml Trizol (Invitrogen, Paisley, UK) in an Eppendorf tube with a power homogeniser. 200µl of chloroform was added to the trizol/ homogenised tissue solution. The tube was capped and shaken vigorously by hand for 15 seconds. The tube was incubated at room temperature for 3 minutes. The tube was centrifuged at 10000 rpm for 15 minutes at 4^{o}C. The aqueous phase was transferred into a new tube.

500µl of isopropyl alcohol was added to the aqueous phase. The tube was incubated at room temperature for 10 minutes. The tube was centrifuged at 10000 rpm for 10 minutes at 4^{o}C. The supernatant was removed with great care taken to avoid inadvertently removing the tiny pellet of RNA. 1ml of 75% EtOH was added to the pellet and the tube shaken by vortex vigorously. The tube was centrifuged at 8000rpm for 10 minutes at 4^{o}C. The supernatant was removed. The RNA pellet was air-dryed for 10 minutes and then dissolved in 50µl of RNAse-free water.

2.9.3. Measuring Optical Density (A 260nm)

99µl of RNAse-free water was put in each of 4 new eppendorf tubes. 1µl of RNA solution from the sample was put in these tubes (100x dilution). 50µl of dilute sample was placed in the spectrophotometer tube and the ultraviolet absorbance was measured at 260nm. The concentration of RNA calculated using the knowledge that an RNA concentration of 40µg/ml has an absorbance of 1.

2.9.4. Assessment of RNA degradation

RNA integrity was tested by running small aliquots of RNA sample on a 1.5% agarose gel to detect 18S and 28S bands. 5M sodium hydroxide was prepared by dissolving 20g NaOH in 100ml of autoclaved deionised water. 20X 3-N-morpholine-propane-sulphonic acid (MOPS) was prepared by adding 83.7g MOPS, 3.72g EDTA and 13.61g Sodium acetate to 200ml autoclaved distilled water. The pH was adjusted to 7.0 using 5M NaOH and the volume was made up to 1L with autoclaved triple-distilled water sterile filtered using a 0.2µm filter. 1X MOPS gel running buffer was prepared by adding 1g of Agarose to a conical flask and adding 85ml of autoclaved distilled water. The flask was heated in a microwave until the agarose had dissolved and then allowed to cool. 5ml of 20X MOPS buffer was added to the flask followed by 5.5ml of formaldehyde (38% stock) and 5µl of Ethidium Bromide. The agarose was poured into a mould whilst still hot and allowed to set for 1 hour. A sample buffer was prepared in an eppendorf tube containing: 10µl of Formamide, 3.5µl of formaldehyde (38% stock) and 1µl of 20X MOPS buffer. 5µg of RNA sample in RNAse-free water was added to the buffer and the total volume made up to 20µl using RNAse-free water. The sample was heated at 55^{o}C for 15 minutes. It was then placed on ice immediately for 2 minutes. The agarose tank was assembled and 1X MOPS gel running buffer was added. 2µl of loading dye solution was added to the sample in the eppendorf tube. 20µl of the sample was loaded onto the gel and run at 32V for 90 minutes. RNA samples were stored at -80^{o}C.

2.10. RIBONUCLEASE PROTECTION ASSAY

2.10.1. Technique of Ribonuclease Protection Assay (RPA)

RPA is a laboratory technique used in biochemistry and genetics to identify individual RNA molecules in a heterogeneous RNA sample extracted from cells. The technique can identify one or more RNA molecules of known sequence even at low total concentration. The extracted RNA is first mixed with antisense RNA or DNA probes that are complementary to the sequence or sequences of interest and the complementary strands are hybridized to form double-stranded RNA (or a DNA-RNA hybrid). The mixture is then exposed to ribonucleases that specifically cleave only *single*-stranded RNA but have no activity against double-stranded RNA. When the reaction runs to completion, susceptible RNA regions are degraded to very short oligomers or to individual nucleotides; the surviving RNA fragments are those that were complementary to the added antisense strand and thus contained the sequence of interest. The protected double-stranded RNA fragments and a small sample of the original probe are then separated on a denaturing polyacrylamide gel, causing the dsRNA to dissociate into single strands. After electrophoresis, the gel is dried, and radioactive signals are measured. The intensity of the radioactive signal generated is directly proportional to the amount of specific target mRNA in the original total RNA sample. Specific bands can be identified for each chemokine on the basis of the migration patterns of the undigested probes. In kits where multiple chemokines are tested, at least one of the plasmids will contain a sequence for the “house-keeping” gene glyceraldehydes-3-phosphate dehydrogenase (GADPH). GADPH is expressed in tissues at a constant level, regardless of the activation status of the cells and, therefore, is used as a control for intersample variation in RNA loading.

Our samples (20µg RNA in each sample) were sent on dry ice to the laboratory of Dr Dai Miyazaki at Tottori University in Japan where the RPA was performed.

The mCK-5c (BD Pharmingen) kit was used for testing for RNA expression of the following mouse chemokines:
Lymphotactin, RANTES, MIP-1β, MIP-1α, MIP-2, IP-10, MCP-1, TCA-3, Eotaxin
Using this kit 20µg of RNA was hybridised overnight at 56^{o}C with 300pg of (α-^{32}P) uridine-triphosphate-labeled antisense riboprobes. Nuclease-protected RNA fragments were resolved on sequencing gels and subjected to autoradiography.

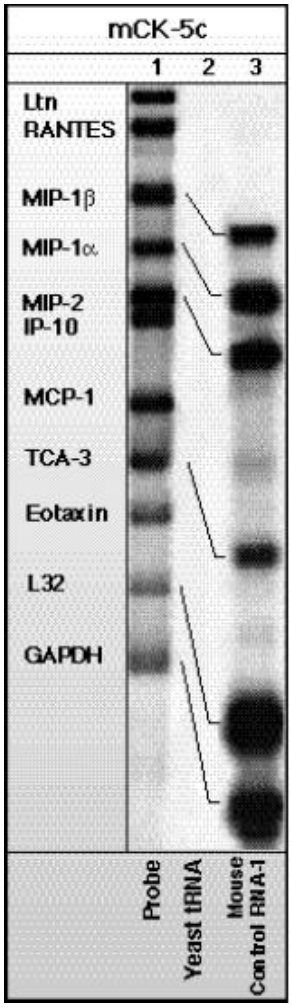


Figure 2.18 Manufacturer's example of a Ribonuclease Protection Assay using the mCK-5c kit

This figure was taken from the BD Pharmingen mCK-5c instruction guide. On the basis of the undigested probes' migration patterns (seen on left), specific bands are identified for each chemokine

2.10.2. Analysis of results of RPA

Protected bands can be observed after exposure of the gel to x-ray film. Specific bands are identified by comparing their indivivual migration patterns to those of undigested probes (Figure 2.18). The bands were quantitated by densitometric analysis (ImageJ, National Institute of Health, Bethesda, USA)(, 2009). Each lane on the gel contained several bands each of which represented RNA of a chemokine gene (Figures 2.18 and 2.19; A). Using a digital photograph of the gel and a gel analysis tool on the ImageJ software, a 2-dimensional plot of the density of each band was constructed (Figure 2.19;C).

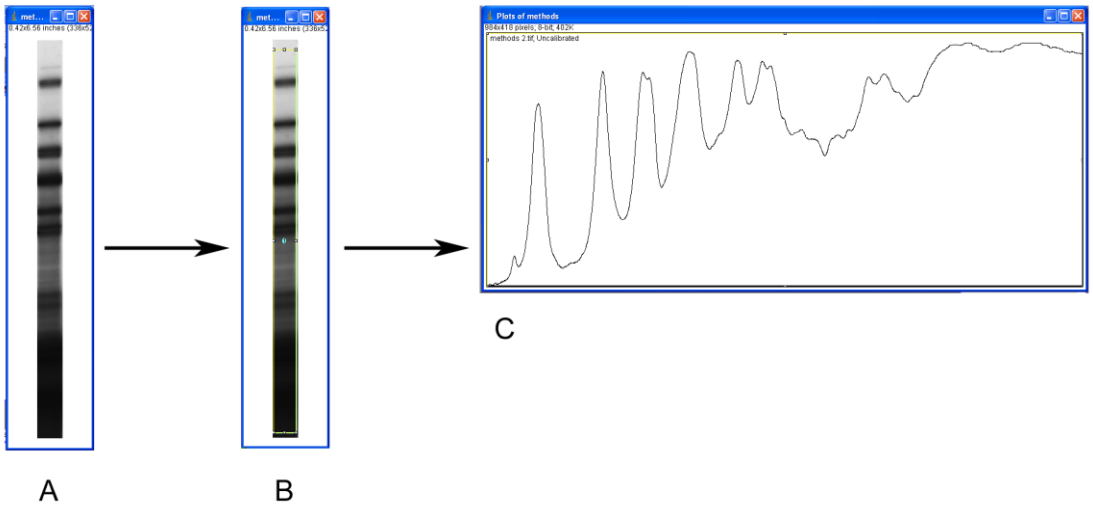


Figure 2.19 Analysis of RPA results using ImageJ

A digital image of the sequencing gel containing chemokine bands is opened in ImageJ (A). The lane of interest is marked using a rectangular box (B; box in yellow). The gel analysis tool on ImageJ then creates a 2-dimensional plot of the density of each band (C). The area under each peak may be calculated.

Each peak on the plot represents a band and the density of the band was calculated by dropping vertical line from each trough and measuring the area under each peak. The areas for each chemokine and GADPH were measured and the chemokine/GADPH ratio was calculated. This ratio, expressed in arbitrary units, reflects the relative abundance of the target mRNA compared with the GAPDH mRNA in each sample. These ratios were then used to determine whether differences in target mRNA expression exist between samples.
This technique has been used by many investigators to quantify the expression of RNA in ocular tissues(Yamagami et al., 1999,Ohta et al., 2000).

2.11. STATISTICS

2.11.1. Actuarial graft survival data

Median graft survival time (MST) was calculated for each group and Kaplan-Meier survival curves were constructed(Kaplan EL and Meier P, 1958). Survival was compared using the log-rank test.

2.11.2. Agreement

Bland-Altman plots were constructed to assess systematic bias in corneal thickness measurements by ultrasound pachymetry(Bland and Altman, 1986).

Pearson’s product-moment correlation coefficient was calculated to measure correlation

2.11.3. Parametric data

The unpaired Student’s T test was used to compare mean values between groups.

2.11.4. Non-parametric data

The Mann-Whitney-U test was used to compare median values between groups.

For each statistical test values of $p < 0.05$ were defined as statistically significant.

3. CHAPTER 3: Effect of allergic conjunctivitis on the immune response to allogeneic donor cornea: Survival, immunohistochemistry and chemokine expression

3.1. INTRODUCTION

Over 100 years after the first human corneal allograft, many of the factors which are likely to lead to graft failure have been identified. Unfortunately, the prognosis for survival in these "high-risk" grafts has improved little in that time. Corneal vascularisation, previous graft failure and glaucoma are all associated with an accelerated rate of graft failure, most commonly due to immune-mediated rejection. Ipsilateral ocular inflammation has also been identified as a predictor of graft failure(Coster and Williams, 2005). Furthermore, the timing of this inflammation appears to be important with perioperative inflammation having the worst prognosis.

The aim of this study was to investigate the effect of a specific type of perioperative ocular inflammation- allergic conjunctivitis- on corneal allograft rejection. Allergic conjunctivitis is important in the context of corneal transplantation for 2 reasons. Firstly, it is the most prevalent form of ocular inflammation in general. It may actually be over-represented in corneal transplant patients given the association between allergic eye disease and keratoconus (Gasset et al., 1978,Harrison et al., 1989,Weed et al., 2008), the commonest indication for corneal transplantation(Cursiefen et al., 1998,Legeais et al., 2001). Secondly, atopy is associated with a skewing of the T helper cell immune responses towards Th2 (Metz et al., 1997,Romagnani, 2000). Alterations in Th1/Th2 bias may influence the immune response to an allograft. Convergent studies have identified the CD4 cell (Th) as the key effector cell in corneal allograft rejection (Yamada et al., 1999a,Haskova et al., 2000). Activated Th cells secrete cytokines which in turn activate and recruit effector cells. Th cells may be classified as Th1 (IL-2, IFN-γ) or Th2 (IL-4, IL-5, IL-10) depending on the profile of their cytokine secretion. Traditionally allograft rejection has been thought to be a Th1-mediated process(Dallman, 1995). This is largely true of unmodified experimental corneal allotransplantation (i.e. where neither the donor nor recipient has undergone any specific preparation, treatment or genetic deletion) (Torres et al., 1996,King et al., 2000). However Th2 and Th1 cells

cross-regulate each other and it has been hypothesized that by enhancing the Th2 response, the Th1 response would be attenuated and graft tolerance achieved (Chen and Field, 1995). Experimental strategies to deviate the immune response towards Th2 in cardiac allografts have had mixed results in terms of allograft survival (Takeuchi et al., 1992,Piccotti et al., 1996,Braun et al., 2000). However one concept has become clear: a Th2-dominant response to alloantigen is capable of graft destruction, possibly via novel effector mechanisms such as eosinophilic infiltration (Chan et al., 1995).

Prior sensitisation to *allergen* has been shown to induce an increased Th2 response to *alloantigen* and, as in other types of allograft, the effect of this on corneal allograft survival have been mixed. In a model of high risk corneal transplantation to a vascularised recipient bed, Th2-bias was reported to extend graft survival (Yamada et al., 1999b). However in a model of normal risk transplantation, in which there is no clinical feature in the recipient or recipient eye conferring high rejection risk, accelerated corneal allograft rejection was found in the setting of allergic conjunctivitis and this was attributed to the Th2-bias induced by systemic sensitisation with allergen (Beauregard et al., 2005).

This chapter examines the effect of perioperative allergic ocular inflammation on allograft survival and on the composition of the inflammatory infiltrate during rejection.

3.2. THE EFFECT OF ALLERGIC CONJUNCTIVITIS ON GRAFT SURVIVAL AND GRAFT-INFILTRATING CELL PHENOTYPE

3.2.1. Research questions

- What is the effect of perioperative allergic conjunctivitis on mouse corneal allograft survival?
- Is any observed effect due to systemic or local influences of the allergic conjunctivitis induction protocol?
- Is any observed effect due to alloantigen-independent inflammation?
- What is the effect of perioperative allergic conjunctivitis on graft-infiltrating cells during rejection?

3.2.2. Experimental methods and design

The techniques of induction of allergic conjunctivitis and corneal transplantation were used in this experiment. These techniques have been described in detail in chapter 2. The protocol for induction of allergic conjunctivitis has been described in detail in chapter 2 and is summarised here in Figure 3.1. The experimental design for this experiment was as described in Figure 3.2. The primary comparison was between naïve recipients of corneal allografts and recipients with perioperative allergic conjunctivitis i.e. recipients that had been sensitised to and challenged with shortragweed pollen at the time of transplantation. These animals were designated $Sens^+$ $Chall^+$. To control for the possible individual confounding effects of systemic sensitisation to shortragweed pollen and local challenge with short ragweed pollen, some animals were sensitised but received no challenge at the time of transplantation ($Sens^+$ $Chall^-$). Others were not sensitized but received a challenge with short ragweed pollen at transplantation ($Sens^-$ $Chall^+$).

Eyes were enucleated at rejection and peroxidase immunohistochemical staining was performed as described in chapter 2 using antibodies to the following antigens:

CD4, CD8, F4/80, MBP

Chapter 2 contains a detailed description of these antibodies and optimised concentrations and conditions for their use in immunohistochemistry.

Digital images of corneal sections were captured using an Olympus digital light microscopy system. Positive-staining cells in the central cornea and the ciliary body were counted. Because rejected corneal allografts demonstrate variable thickness due to oedema, it was not appropriate to count the number of cells per unit area. Instead, the number of positive cells throughout the full thickness of a x100 field of the central stroma of each section was counted. Cells were counted in 3 sections per rejected graft. At least 5 grafts were examined in each group. The mean number of cells in the grafts was calculated.

Sections of the ciliary body were imaged by light microscopy and their cross-sectional areas measured using image analysis software (Soft Imaging System GnbH, Munster, Germany). The number of positive-staining cells in each ciliary body section was counted using high magnification and expressed as cells / 0.1 mm^2. Cells were counted in 3 sections per eye. At least 5 eyes were examined in each group. The mean number of cells in each ciliary body was calculated.

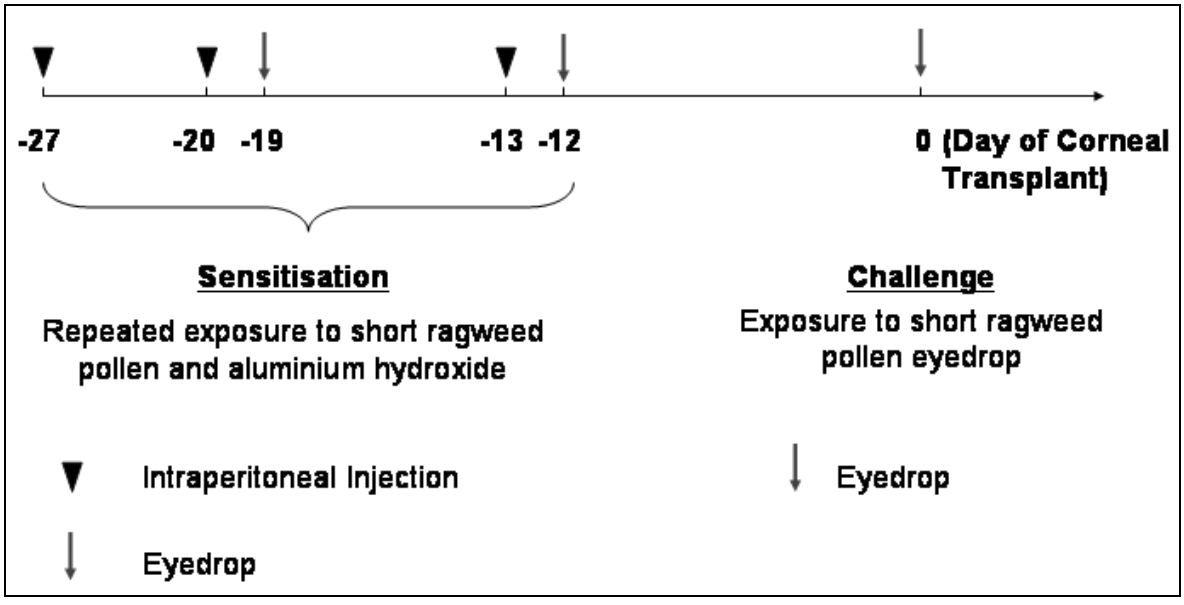


Figure 3.1 Protocol for induction of allergic conjunctivitis in mice

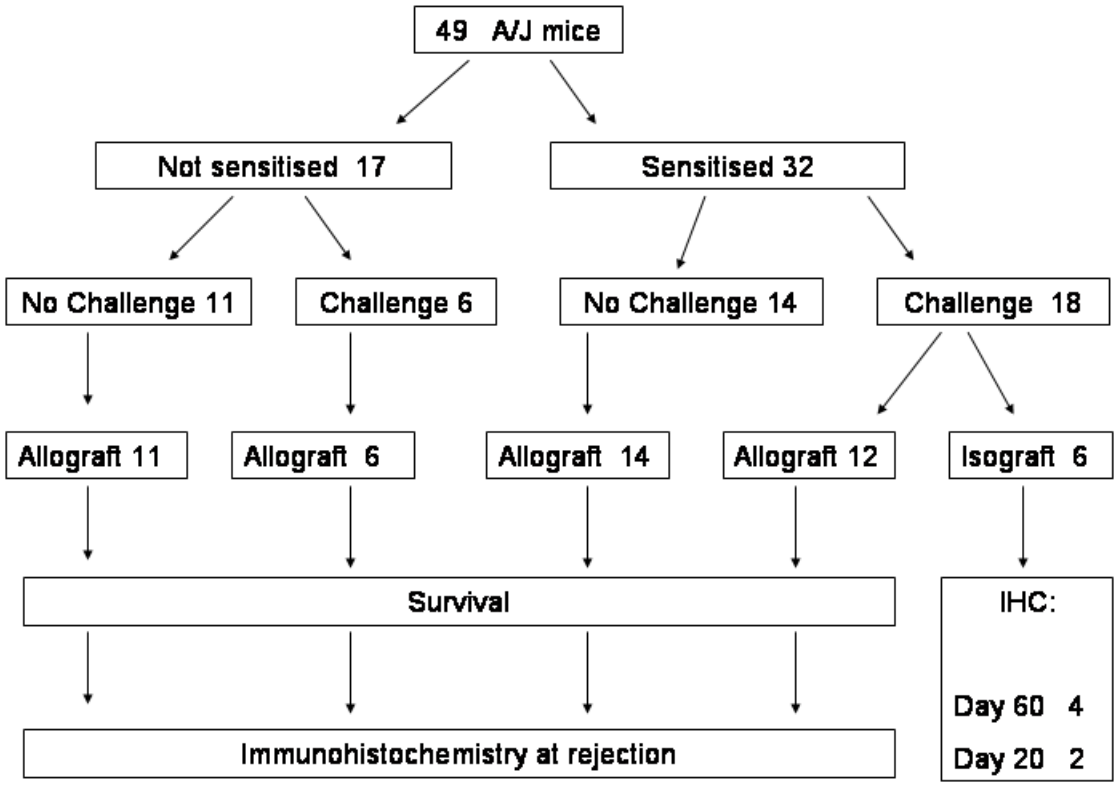


Figure 3.2 Experimental design part 1

3.2.3. Results

3.2.3.1. Survival of corneal allografts

Baseline survival of C57BL/6 allografts in naïve A/J mice was first established. By 60 days 73% of these naïve A/J mice rejected their allografts with a median survival time (MST) of 36 days. Allografts were then performed in A/J mice that had been sensitised to SRW pollen. Immediately following transplantation, these sensitised mice were challenged with SRW eyedrops in the transplant recipient eye, to induce allergic conjunctivitis. These sensitised & challenged ($Sens^+$ $Chall^+$) A/J mice rejected 100% of their allografts with a significantly lower MST of 16 days ($p<0.001$). Next corneal allografts were performed in sensitised mice which were then mock-challenged with PBS in the graft recipient eye. These mice, which were sensitised but not challenged ($Sens^+$ $Chall^-$), rejected their grafts in similar tempo to naïve mice, with 71% of grafts rejected with MST 32 days (Figure 3.3). This was significantly slower than the rate of rejection in $Sens^+$ $Chall^+$ mice. ($p=0.001$). Mice that were not sensitised but were challenged at the time of transplantation rejected 100% of grafts at an MST of 31 days. Median survival times of corneal grafts in each group are shown in Table 3.1. P values for pairwise comparison of graft survival between groups using the Log Rank test are shown in Table 3.2.

These data suggest that the presence of perioperative allergic conjunctivitis significantly decreases the time to subsequent allograft failure and that this effect due more to local conjunctival inflammation than to any systemic effects of the allergic conjunctivitis induction protocol.

3.2.3.2. Survival of corneal isografts

To establish whether the failure of grafts in allergic mice was primarily due to a specific response against alloantigens or non-specific allergic inflammation, syngeneic corneal grafts were performed in sensitised A/J mice which were then challenged with SRW eyedrops in the graft eye to induce allergic conjunctivitis. 100% of these syngeneic grafts survived for 60 days. This indicates that the accelerated graft failure seen in recipients with allergic conjunctivitis is due to alloantigen-dependent inflammation i.e immune rejection.

Donor	**Recipient**	**Median Survival Time (days)**
C57BL6	A/J (Naïve)	36
C57BL6	A/J ($Sens^{+}Chall^{+}$)	16
C57BL6	A/J ($Sens^{+}$ $Chall^{-}$)	32
C57BL6	A/J ($Sens^{-}$ $Chall^{+}$)	31
A/J	A/J ($Sens^{+}Chall^{+}$)	>60

Table 3.1 Median survival times of corneal grafts in each group

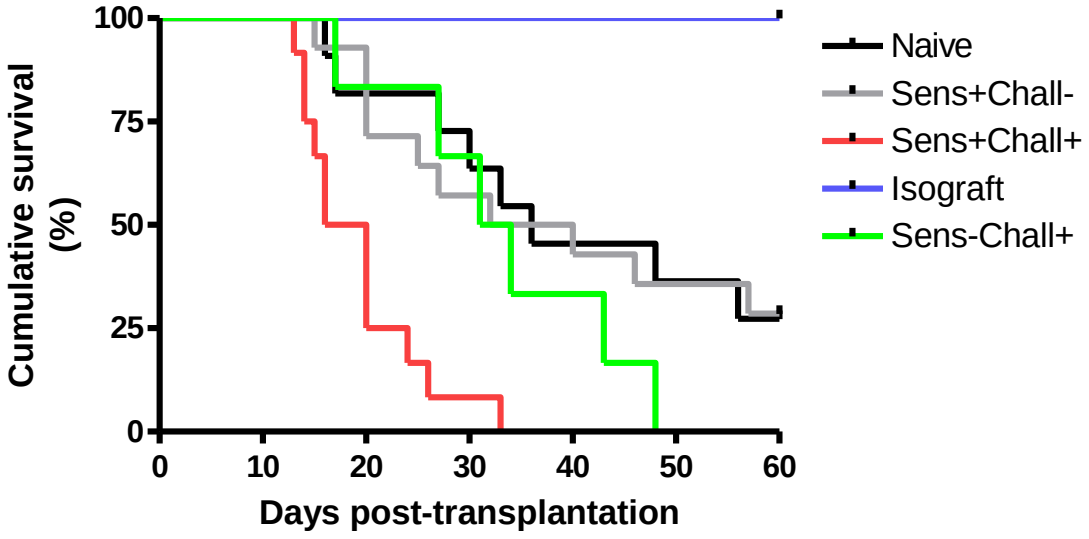


Figure 3.3 Actuarial corneal transplant survival

Allografts in Sens^{+} Chall^{+} eyes (red curve) (n=12; MST = 16 days) were rejected at a significantly faster tempo than either those in naïve eyes (black curve) (n=11; MST=36days), those in Sens^{+} Chall^{-} eyes (grey curve) (n=14; MST=32) or those in Sens^{-} Chall^{+} eyes (green curve) (n=6; MST=31 days). Isografts in sensitised and challenged eyes (blue line) (n=4) survived beyond 60 days.

	Naive	Sens^{+} Chall^{+}	Sens^{+} Chall^{-}	Sens^{-} Chall^{+}
Naive		<0.001	0.941	0.218
Sens^{+} Chall^{+}	<0.001		0.001	0.006
Sens^{+} Chall^{-}	0.941	0.001		0.283
Sens^{-} Chall^{+}	0.218	0.006	0.283	

Table 3.2 P values for pairwise comparison of graft survival between groups using the Log Rank test.

3.2.3.3. Immunohistochemistry of rejected allografts

The phenotypes of graft-infiltrating cells were characterised using immunohistochemistry and comparisons made between rejected grafts in naïve, $Sens^+$ $Chall^+$ and $Sens^+$ $Chall^-$ mice. There were no significant differences between in the numbers of $CD4^+$, $CD8^+$ and $F4/80^+$ cells infiltrating the grafts at the time of rejection in all groups (Fig 3.4). Within each group, there were no significant differences in numbers of graft-infiltrating $CD4^+$, $CD8^+$ and $F4/80^+$ cells.

MBP^+ cells were found consistently in rejected grafts in $Sens^+$ $Chall^+$ mice but were seldom found in rejected grafts in naïve mice or $Sens^+$ $Chall^-$ mice (Figure 3.5). Despite the significant association ($p = 0.01$) between the presence of perioperative allergic conjunctivitis and the presence of an eosinophilic infiltrate at the time of graft rejection , the number of graft-infiltrating eosinophils in $Sens^+$ $Chall^+$ eyes was significantly lower than those of $CD4^+$ cells, $CD8^+$ cells or macrophages (Figure 3.4).

MBP^+ cells were also seen consistently in the uveal tract of $Sens^+$ $Chall^+$ eyes at the time of rejection ($p=0.003$). They were not found in rejected grafts in $Sens^+$ $Chall^-$ eyes and were seldom seen in naïve eyes (Figure 3.6).

At the time of rejection eosinophils were seen in the conjunctiva of $Sens^+$ $Chall^+$ eyes but were not seen in $Sens^+$ $Chall^-$ or naïve eyes (Figure 3.7).

No infiltrating cells were seen in the sclera of corneal graft recipients. In particular no eosinophils were seen in the sclera during rejection in $Sens^+$ $Chall^+$ recipients indicating that this model does not provide model of allograft-induced atopic sclerokeratitis (Figure 3.7; D).

3.2.3.4. Immunohistochemistry of isografts

Eosinophils were also seen, at 60 days, in the conjunctiva of Sens$^+$ Chall$^+$ eyes that had received isografts (Fig. 3.7). However no eosinophils were seen in the isografts in Sens$^+$ Chall$^+$ eyes at 60 days nor were they seen in the ciliary body of these eyes. An additional group of isografts was performed in Sens$^+$ Chall$^+$ eyes and these eyes were removed for immunohistochemistry on post-operative day 20. In this group also eosinophils were found in the conjunctiva but none were seen infiltrating the graft or ciliary body indicating that eosinophils in the graft/ciliary body during allograft rejection are unlikely to represent part of the wound-healing, innate inflammatory respone.

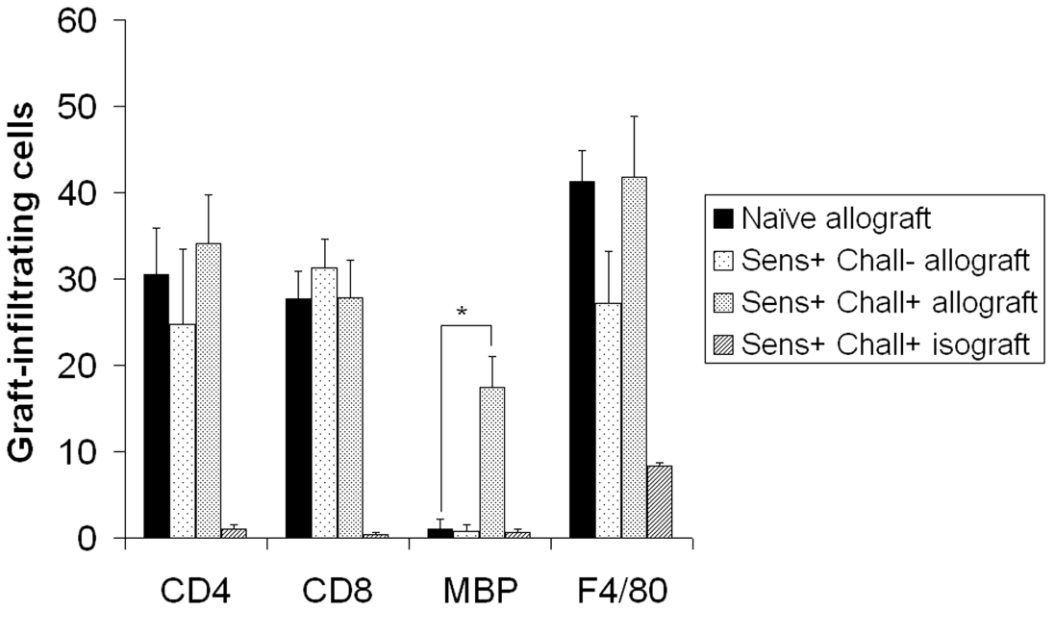


Figure 3.4 Immunohistochemistry of rejected corneal grafts

The number of cells in a x100 field staining for CD4, CD8, MBP (eosinophils) and F4/80 (macrophages) were counted and the mean +/- SE are shown. CD4$^+$ cells, CD8$^+$ cells and macrophages were seen consistently in all allograft groups. Eosinophils were seen predominantly in allografts in eyes that had perioperative allergic conjunctivitis but not in isografts. * $p < 0.01$

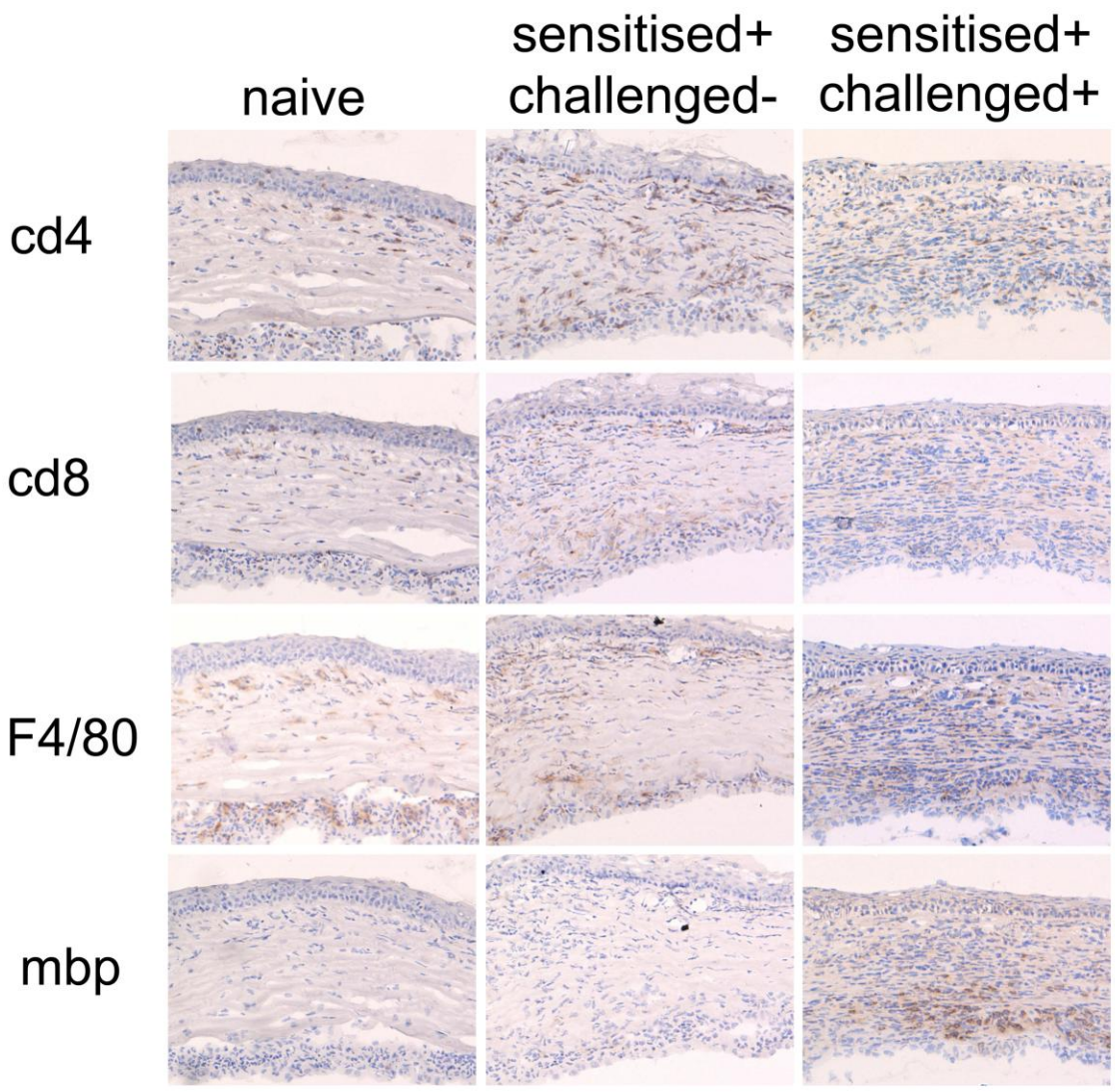


Figure 3.5 Immunohistochemical staining in rejected corneal grafts

Photomicrographs of corneal sections from rejected grafts stained by peroxidase immunohistochemistry and imaged at an original magnification of x200. Eosinophils are seen in $Sens^{+}$ $Chall^{+}$ eyes but not in naïve eyes or $Sens^{+}$ $Chall^{-}$ eyes. $CD4^{+}$, $CD8^{+}$ and $F4/80^{+}$ cells are seen in all groups.

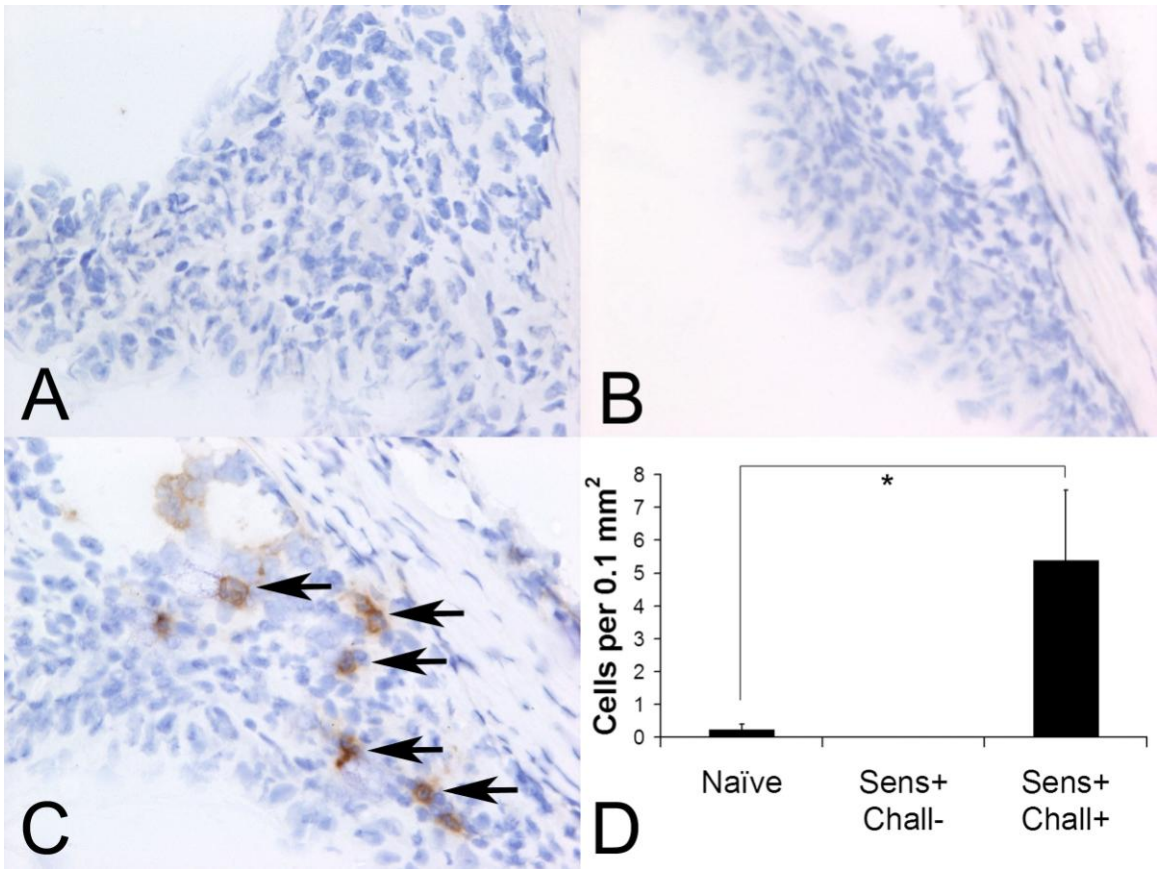


Figure 3.6 5 Immunohistochemical staining for Major Basic Protein in ciliary body

Eosinophils were seldom seen in the ciliary body of naïve eyes (A) during corneal allograft rejection and were never seen in $Sens^{+}$ $Chall^{-}$ eyes (B). Eosinophils (arrows) were seen in the ciliary body of $Sens^{+}$ $Chall^{+}$ eyes during rejection (C). The histogram (D) depicts the number of MBP-positive cells per $0.1mm^2$ of ciliary body. * $p< 0.05$.

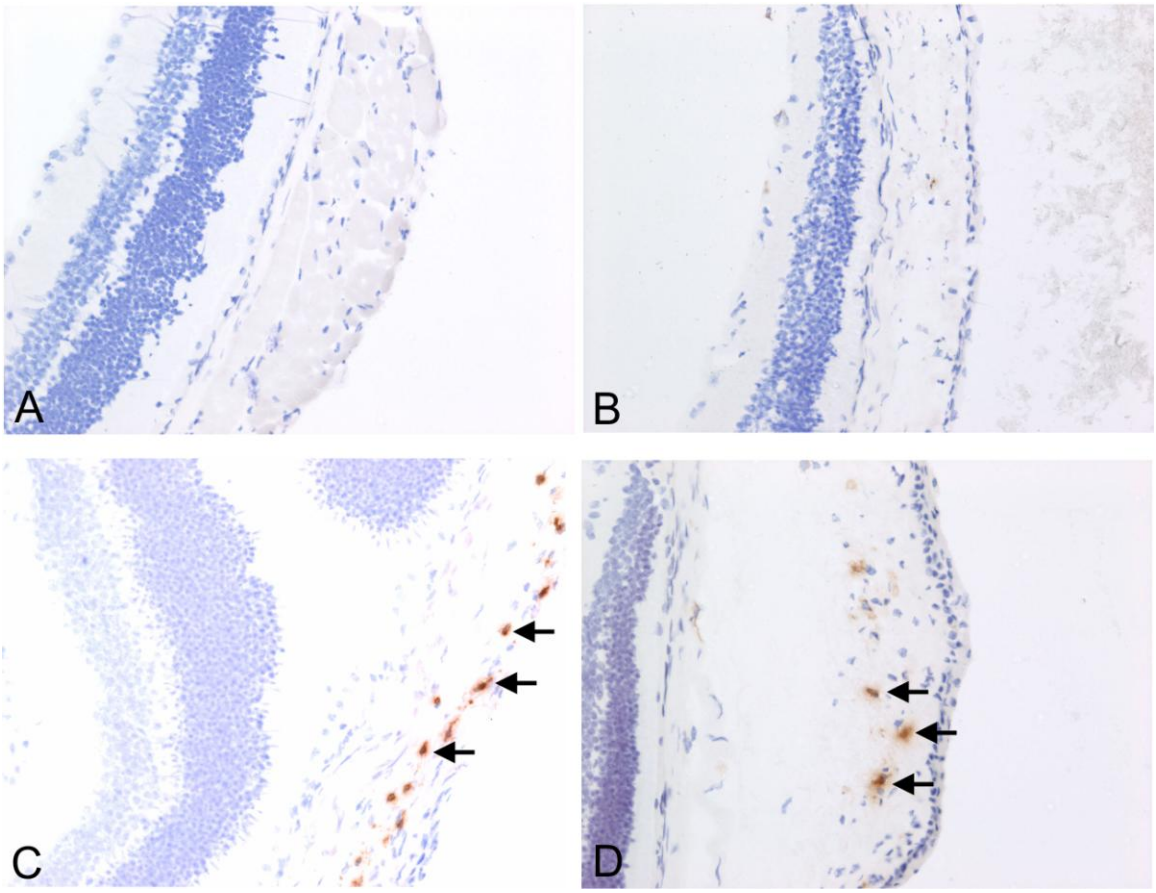


Figure 3.7 Immunohistochemical staining for Major Basic Protein in conjunctiva.

At the time of allograft rejection no eosinophils are seen in naïve eyes (A) or $Sens^{+}$ $Chall^{-}$ eyes (B). Eosinophils (arrows) are seen in the conjunctiva (but not the sclera) of $Sens^{+}$ $Chall^{+}$ eyes (C). Eosinophils are also seen in the conjunctiva of $Sens^{+}$ $Chall^{+}$ eyes 60 days after receiving a syngeneic graft (D).

3.3. THE EFFECT OF SENSITISATION ON THE HOST CORNEA

3.3.1. Research question

Does the allergic conjunctivitis protocol alter the number of antigen-presenting cells and lymphatic vessels in the cornea prior to transplantation and final challenge?

3.3.2. Experimental methods and design

The experimental design for this experiment was as described in Figure 3.8. Mice were sensitised to shortragweed pollen as described in detail in Chapter 2. Eyes were enucleated and fluorescence immunohistochemical staining was performed as described in detail in Chapter 2 using the following antibodies:

PE-conjugated anti-CD11b (macrophage/ neutrophil/ dendritic cell marker)
PE-conjugated anti-CD11c (dendritic cell marker)
Anti-LYVE-1
Alexa 488-conjugated Donkey anti-Rat IgG

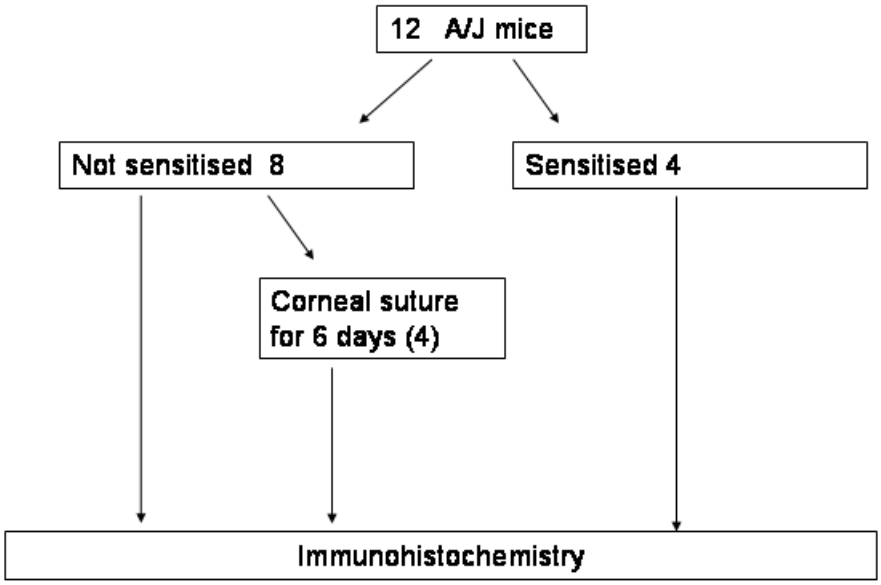


Figure 3.8 Expermental design part 2

3.3.3. Results

Large numbers of infiltrating $CD11b^+$ and smaller numbers of $CD11c^+$ cells were seen in the cornea 6 days following placement of corneal sutures. LYVE-1 staining was also consistently seen in the corneal stroma in these eyes. By comparison, few $CD11b^+$ and $CD11c^+$ cells and no LYVE-1 staining were seen in the cornea in either normal eyes or in $Sens^+$ $Chall^-$ eyes. (Figure 3.9)

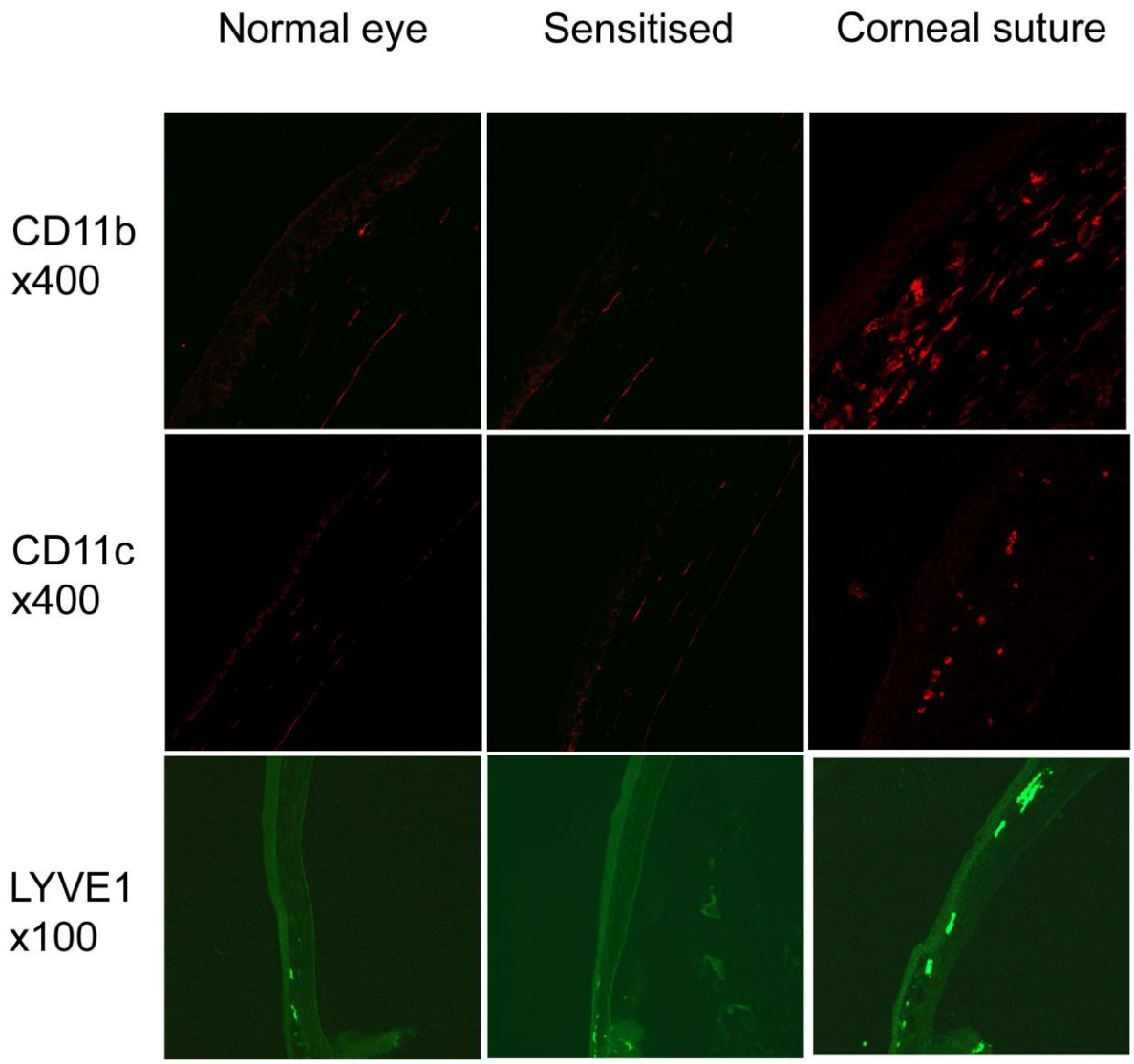


Figure 3.9 Effect of sensitisation on the host cornea

Although the sensitization protocol does involve topical exposure to short ragweed it does not alter the corneal stromal content of dendritic cells (CD11c) or macrophages (CD11b) and does not induce the formation of new lymphatics (LYVE-1). This is in contrast to the established model of high-risk corneal transplantation (suture-induced corneal inflammation).

3.4. THE EFFECT OF ALLERGIC CONJUNCTIVITIS ON CHEMOKINE EXPRESSION AFTER CORNEAL TRANSPLANTATION

3.4.1. Research question

Does perioperative allergic conjunctivitis alter the expression of the chemokines Eotaxin and RANTES during corneal allograft rejection?

3.4.2. Experimental methods and design

The techniques of induction of allergic conjunctivitis and corneal transplantation were used in this experiment. These techniques have been described in detail in chapter 2. The experimental design for this experiment was as illustrated in Figure 3.10. Eyes were enucleated at day 12 post-transplantation and RNA was extracted as described in Chapter 2, the RNA from 3 eyes at a time being pooled for each RNA sample. RNA was analysed by Ribonuclease Protection Assay as described in Chapter 2. This technique provided data not only on Eotaxin and RANTES but also on MIP-1α, MIP-1β, MIP-2, MCP-1, IP-10 and Lymphotactin. Deciding on the best timepoint for RNA extraction was difficult. King showed that peak expression of chemokines in the rat cornea co-incided with the clinical onset of rejection(King et al., 2000). In cardiac transplantation increased chemokine expression was noted just before onset of rejection(Fairchild et al., 1997). Pillai and colleagues, studying corneal allografts in mice, later found a peak in chemokine expression also just before onset of clinical rejection(Pillai et al., 2008a). Both King and Pillai showed peaks of post-transplant chemokine expression at days 11-14 but they used very predictable donor recipient combination in their respective models of corneal allograft rejection where all grafts were rejected within a day or two of each other. My models were quite different with onset of rejection ranging over 43 days in naïve recipients and over 20 days in recipients with allergic conjunctivitis. This meant that while one graft was rejecting and presumably expressing increased levels of rejection-

associated chemokines another may be healthy and expressing low levels of chemokines. It was not possible to extract the RNA on the day that rejection was observed because the RNA from 3 eyes had to be pooled for each sample and it was unlikely that 3 eyes would consistently reject on the same day. One approach, used by Yamagami et al (Yamagami et al., 1999) was to extract the RNA at the timepoint where 50% of grafts had rejected i.e. the median survival time. In my groups this would have meant extracting RNA at different timepoints (36 days in naïve recipients and 16 days in recipients with allergic conjunctivitis) and this did not seem to be scientifically correct. Onset of clinical rejection was first observed at day 13 in recipients with allergic conjunctivitis and at day 17 in naïve recipients. It was therefore decided to extract the RNA at the same timepoint (day 12) in all groups. Isografts were performed in naïve recipients and recipients with allergic conjunctivitis as additional controls.
RNA extraction, Ribonuclease Protection Assay and analysis of RPA results were performed as described in detail in Chapter 2.

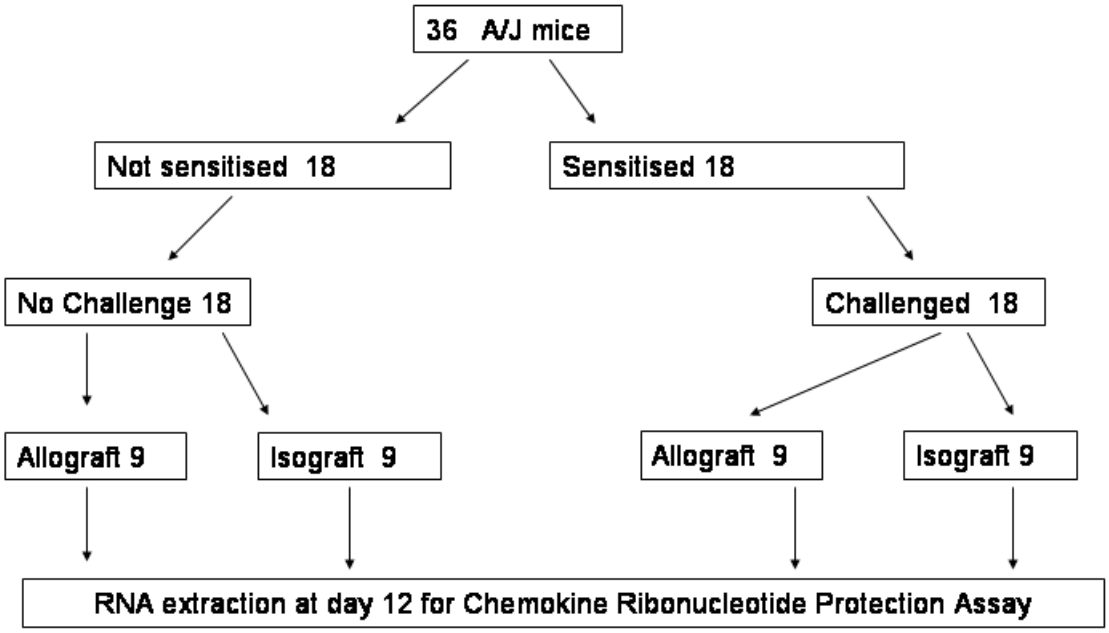


Figure 3.10 Experimental design part 3

3.4.3. Results

The RNA from 3 animals was pooled to give 12 samples (9 eyes per group = 3 samples per group). At least 20 µg of RNA would be required for analysis of each sample. Greater than 20 µg of RNA was extracted from 10 of the 12 samples (Table 3.3). The presence of distinct 28S and 18S bands on gel analysis of the extracted RNA indicated that the extracted RNA was reasonably intact (Figure.3.11)

		Sample	Quantity of RNA (µg)
Allografts	$Sens^+$ $Chall^+$	1	31.65
		2	39.04
		3	0
	Naive	4	57.45
		5	53.66
		6	43.67
Isografts	$Sens^+$ $Chall^+$	7	72.93
		8	51.77
		9	69.8
	Naive	10	18.57
		11	40.93
		12	21.67

Table 3.3 Quantity of RNA extracted from mouse eyes

Note each sample contained the pooled RNA from 3 whole eyes from which the conjunctiva had been removed.

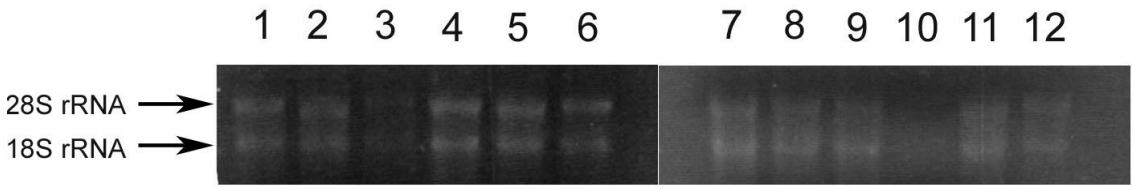


Figure 3.11 Integrity of RNA extracted from mouse eyes

The samples are numbered 1-12 and these numbers correspond to those of the samples described in table 3.3. Each sample contains the RNA extracted from 3 whole eyes at day 12 post corneal transplantation. The presence of distinct 28S and 18S bands on gel analysis of the extracted RNA indicated that the extracted RNA was reasonably intact. Samples 3 and 10 contained little RNA (see table 3.3) and have correspondingly low band visibility.

Chemokine results were obtained for 10 samples each of which contained combined RNA from 3 eyes (10 lanes; 2 x allergic allograft, 3 x naïve allograft, 3 x allergic allograft, 2 x naïve isograft). The output of the ribonuclease protection assay is a photo of a gel. Bands in each lane represent chemokine RNA. Figure 3.12 shows a representative lane from each group. The densities of the bands were calculated for each chemokine as described in Chapter 2 and the mean value for each group was calculated.

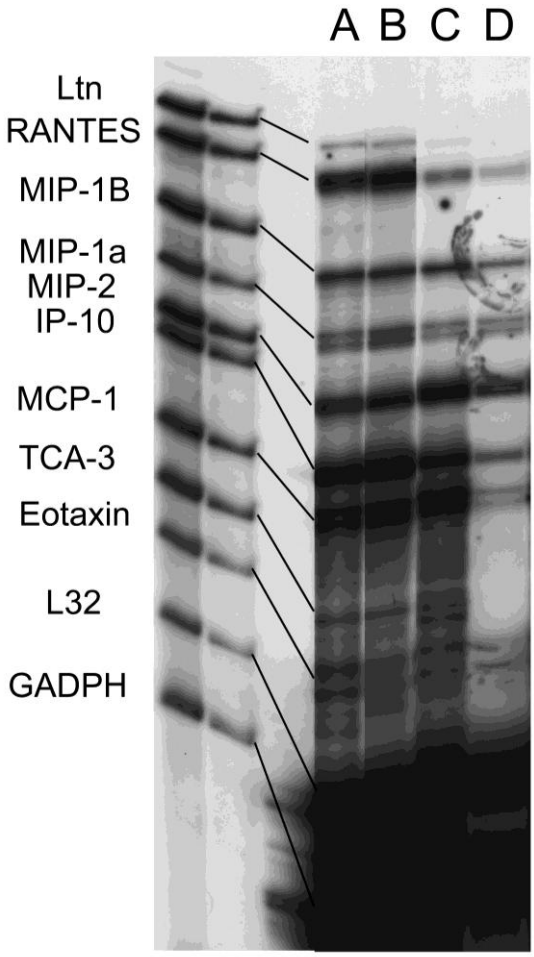


Figure 3.12 Chemokine gene expression after corneal transplantation in A/J hosts

Autoradiography. Twenty micrograms of pooled RNA was applied in each lane. On the basis of the undigested probes' migration patterns (seen on left), specific bands were identified for each chemokine: *lane A*, representative allergic allografts; *lane B*, representative naïve allografts; *lane C*, representative allergic isografts; *lane D*, representative naïve isografts.

As discussed in chapter 2 the quantification of RNA expression using densitometric analysis is only loosely quantitative especially for the 3 chemokines at the lower end of each lane where the image was relatively overexposed. Therefore when comparing between groups (lanes) I chose to recognise differences in the mean chemokine expression that were a multiple of 2 or

greater to denote a significant difference. The results are described for each chemokine on an individual basis and are illustrated in Figure 3.13.

Lymphotactin

Lymphotactin expression was increased in naïve recipients of allografts compared to naïve recipients of isografts, allergic recipients of isografts and allergic recipients of allografts.

RANTES

RANTES expression was increased in naïve recipients of allografts compared to naïve recipients of isografts, allergic recipients of isografts and allergic recipients of allografts.

MIP-1β

There were no clear differences in expression of MIP-1β across the four groups.

MIP-1α

There were no clear differences in expression of MIP-1α across the four groups.

MIP-2

There were no clear differences in expression of MIP-2 across the four groups.

IP-10

IP-10 expression was increased in naïve recipients of allografts compared to naïve recipients of isografts, allergic recipients of isografts and allergic recipients of allografts.

MCP-1

There were no clear differences in expression of MCP-1 across the four groups.

TCA-3

There were no clear differences in expression of TCA-3 across the four groups.

Eotaxin

There were no clear differences in expression of Eotaxin across the four groups.

The primary question in relation to chemokine expression was whether increased expression of chemokines chemotactic for eosinophils (Eotaxin and RANTES) would be increased in allograft rejection in eyes with perioperative allergic conjunctivitis compared with rejection in naïve eyes. In allergic allografts there was no obvious difference in expression of eotaxin compared with naïve allografts at day 12. Allergic allografts expressed obviously *less* RANTES than naïve grafts at day 12.

In animals with perioperative allergic conjunctivitis there were no clear differences in chemokine expression at day 12 between recipients of allografts and recipients of isografts (Figure 3.13). These data suggest that either perioperative allergic conjunctivitis inhibits chemokine expression during graft rejection or that the day 12 timepoint missed any rejection-associated increase in chemokine expression in eyes with perioperative allergic conjunctivitis.

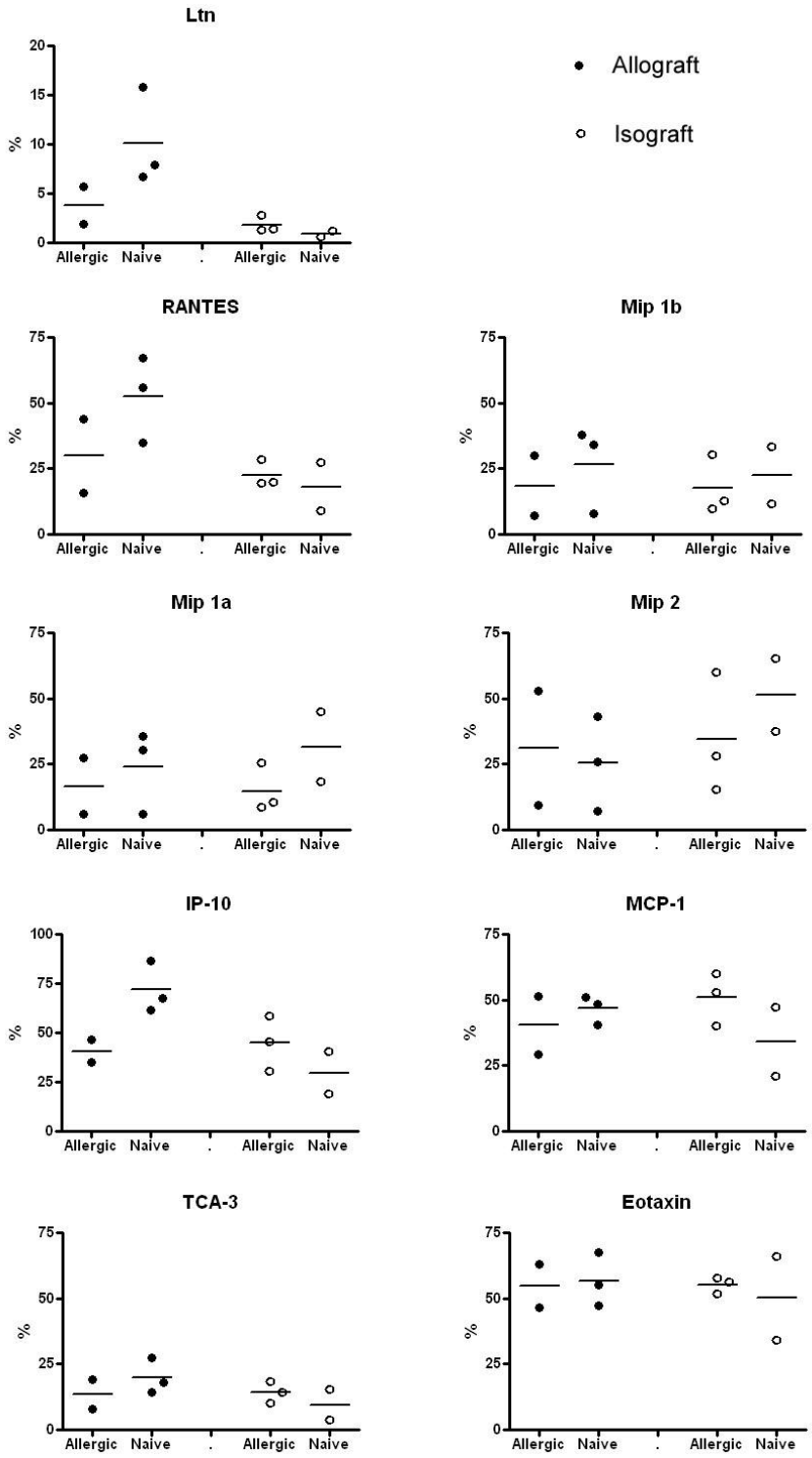


Figure 3.13 Chemokine gene expression after corneal transplantation in A/J hosts: Normalized densitometric analyses

The y axis represents arbitrary units expressed as a percentage of GADPH expression.

3.5. DISCUSSION

3.5.1. Effect of allergic conjunctivitis on graft survival

Data from large cohort outcome studies have demonstrated the negative impact on graft survival of ocular inflammation at the time of corneal transplantation(Williams et al., 1989). The results of our study are consistent with these data and show that allergic conjunctivitis, in particular, accelerates corneal allograft rejection. This result is also consistent with the finding by Beauregard and coworkers of an increased tempo of corneal graft rejection in their model of chronic post-operative allergic conjunctivitis(Beauregard et al., 2005). Whereas Beauregard continued to challenge his graft recipients after transplantation I performed a single challenge at the time of surgery. These data indicate that allergic inflammation in the perioperative period alone is sufficient to shorten graft survival.

3.5.2. The effect of allergy on the composition of graft infiltrate at rejection

The immune response to alloantigen comprises an afferent and an efferent arm. In the afferent arm antigen-presenting cells (APC) travel from the graft bearing alloantigen to regional lymph nodes where it is presented to T cells. The efferent arm culminates in infiltration and destruction of the graft by a variety of effector cells. Our finding of $CD4^+$ cells, $CD8^+$ cells and macrophages in rejected grafts is consistent with previous reports in mouse and human corneal transplantation(Larkin et al., 1997a,Kuffova et al., 2001). No inflammatory response was seen in the sclera of allergic recipients of corneal allografts suggesting that this does not provide an experimental model of atopic sclerokeratitis which is a well-recognised entitiy in atopic patients post-transplantation(Lyons et al., 1990).

We have shown that perioperative allergic conjunctivitis influences the effector arm of the immune response in that it is associated with an eosinophilic infiltrate during graft rejection. Corneal graft infiltration by eosinophils has been previously described in rejected human allografts in patients with allergic conjunctivitis (Hargrave et al., 2003) and in a mouse model of allergic conjunctivitis(Beauregard et al., 2005). Eosinophilic infiltration is a prominent feature of unmodified rejection in corneal and pancreatic xenotransplants (Simeonovic et al., 1999b,Tanaka et al., 2005,Larkin et al., 1995). In animal models of skin and cardiac allotransplantation eosinophilic infiltration is seen characteristically in Th2-biased animals(Braun et al., 2000,Le et al., 1999b).

Three questions need to be addressed regarding eosinophils: *(i)* are they specifically recruited to the cornea during graft rejection? *(ii)* Are they contributing to graft destruction? And *(iii)* are they responsible for the increased tempo of graft rejection?

Eosinophils entering the cornea and anterior chamber in allergic eyes appear to do so as part of the specific response to alloantigen, supported by the observation of no eosinophils in isograft recipient eyes with allergic conjunctivitis despite their presence in the conjunctiva. Eosinophils themselves are part of the innate immune system and do not have specificity for alloantigen. They may however be recruited by Th2-biased CD4+ cells with specificity for alloantigen. Prior sensitisation with allergen as in our model has been shown to bias the animal toward Th2 response (even to an unrelated antigen)(Yamada et al., 1999b,Beauregard et al., 2005). Therefore one explanation for the presence of eosinophils is that in animals that have previously been sensitized to allergen, exposure of CD4 cells to alloantigen induces a TH2 phenotype which recruits eosinophils to the graft during rejection. If this was the case eosinophils may be expected in the graft infiltrate of rejected grafts in sensitized recipients who were not challenged ($sens^{+}chall^{-}$) as these animals would also be Th2-biased. However no eosinophils were seen in rejected grafts of this group in my experiment. Beauregard performed a similar experiment and also found no

eosinophils in this group. It therefore appears that prior sensitization alone is not sufficient to produce a graft infiltrate of eosinophils but that active allergic conjunctivitis at the time of transplantation is required.

$Sens^+$ $Chall^+$ graft recipients have many eosinophils in their conjunctiva. During graft rejection one possible route of alloreactive cell trafficking to graft stroma is from the surrounding conjunctiva (the "side door"). Therefore another explanation for their presence in the cornea during rejection is that the conjunctiva, at the time of graft rejection, still contains eosinophils following the allergen exposure and that these eosinophils enter the cornea from the conjunctiva during graft rejection along with other mediators of rejection. Reports on the longevity of the late cellular response in animal models of allergic conjunctivitis suggest it lasts 2-3 days(Choi and Bielory, 2008). In our model, first rejection was seen at 15 days post-transplantation (and post allergen exposure). Immunohistochemical analysis of the conjunctiva during rejection showed that eosinophils were present in the conjunctiva during rejection (i.e at 15 + days post-allergen exposure) which suggests that the cellular mediators of the late phase of allergic conjunctivitis remain in the conjunctiva for longer than has been thought. If these eosinophils were simply "dragged in" to the cornea from the conjunctiva during rejection along with other more specific cellular mediators, we would not expect to see eosinophils in the anterior uveal tract (which provides an alternative access to the graft endothelium via the anterior chamber) as even in allergic conjunctivitis these tissues do not contain eosinophils. However we do see eosinophils in the ciliary body during rejection in recipients with allergic conjunctivitis.

The capacity of eosinophils in parasitic and allergic inflammation to initiate and sustain an inflammatory response is largely due to the release of cationic proteins including major basic protein, eosinophil cationic protein, eosinophil peroxidase and eosinophil-derived neurotoxin (Rothenberg and Hogan, 2006). These proteins can directly injure mammalian cells and can induce cytokine and chemokine release from bystander cells. Eosinophils certainly have the

capacity to injure the graft but their importance as effector cells in corneal allograft rejection remains undetermined. That said, of the CD4 cells, CD8 cells and macrophages that have been consistently found in the infiltrate of rejected grafts, only CD4 cells have been shown to play an essential role in the rejection process(Yamada et al., 1999a). Although graft-infiltrating eosinophils were seen exclusively in the context of allergic conjunctivitis, we found their absolute number to be less than those of CD4 cells, CD8 cells or macrophages.
These data suggest to me that eosinophils enter the cornea/anterior chamber as part of the acquired immune response to alloantigen but that for this to happen the animal must be both sensitized to allergen and challenged with the allergen at the time of surgery. The number of eosinophils being much less that the other cellular mediators of rejection, it is not plausible that these cells are alone responsible for the accelerated rate of rejection. However they may represent a change in the effector component of the acquired immune response brought about by the presence of allergic conjunctivitis at the time of corneal transplantation.

3.5.3. Local versus systemic effects of allergy

Animals in the model of allergic conjunctivitis we report underwent two interventions, either of which could in theory have influenced graft survival. The preliminary sensitisation of animals to SRW skews subsequent T cell cytokine responses towards Th2 as shown by Yamada (Yamada et al., 1999b) .
Subsequent challenge with topical SRW induced local ocular inflammation.
The sensitisation process does involve some instillation of allergen on the ocular surface. In our model there was no exposure to allergen in the 2 weeks prior to the final challenge. The conjunctiva of these animals appears normal clinically prior to challenge. Histological examination of the cornea prior to final allergen challenge reveals no difference to a naïve cornea in terms of lymphatic vessel and cells with antigen-presenting capacity ($CD11b^+$ and $CD11c^+$).

It is of interest that animals sensitised to SRW but not challenged at the time of transplantation rejected allografts at a similar tempo to naïve animals. This suggests that local inflammation rather than any change in Th2 bias is responsible for the increased tempo of allograft rejection following perioperative allergic conjunctivitis because if systemic changes induced by the sensitisation process were responsible for the accelerated rate of rejection we would have expected these animals to reject their grafts at a different rate to naïve animals. For reasons that are not clear, this finding is in contrast with the report by Beauregard et al that, in their model of allergic conjunctivitis, the increased tempo of allograft rejection was attributable to sensitisation to allergen rather than local inflammation(Beauregard et al., 2005).

Beauregard found that the tempo of rejection was accelerated in allograft recipients with active allergic conjunctivitis ($Sens^+$ $Chall^+$) as compared to naïve recipients and these results were similar to ours. Their allergic conjunctivitis induction protocol differed slightly from ours in that we challenged the conjunctiva with SRW only once (immediately following transplantation) whereas they challenged with allergen throughout the post-transplantation period of observation. Therefore our study adds new information that allergic inflammation at the time of surgery is sufficient to accelerate subsequent rejection.

The fundamental difference between our results and those of Beauregard however lies in the groups of animals that were sensitised but not challenged ($Sens^+$ $Chall^-$). In our study these animals rejected grafts at a similar rate to naïve animals. In Beauregard's they rejected grafts at an accelerated rate similar to animals that were challenged with allergen which seems to suggest that the accelerated rate of rejection is due to systemic changes induced by the sensitization protocol.

The question of the differential effects of local and systemic atopic phenomena on corneal graft rejection is a key one.

Beauregard et al report increased expression of IL-4 and Il-5 (Th2 cytokines) by T cells of “Atopic” graft recipients in response to alloantigen but it is not clear from their data whether this refers to $Sens^+$ $Chall^+$ recipients, $Sens^+$ $Chall^-$ recipients or both. Interestingly, they, like us, found no eosinophils in rejected grafts of sensitized recipients who were not challenged with allergen suggesting that the Th2 effector mechanisms are more marked in the presence of allergic conjunctivitis than in sensitized animals without allergic conjucntivitis.

Differences in the models of allergic conjunctivitis may explain in part the variance in results: a different strain of graft recipient mouse was used in the experiments we report. We used A/J mice as recipients whereas they used BALB/c mice. As outlined in the introduction to the thesis immune responses may be quite different in different strains of mice even under similar conditions(Yamada et al., 2009).
Our experimental protocols also differed. We designed $Sens^+$ $Chall^+$ and $Sens^+$ $Chall^-$ groups to represent, as closely as possible, the clinical picture seen in allergic conjunctivitis patients with and without active or uncontrolled conjunctival disease. Therefore our $Sens^+$ $Chall^-$ animals received one mock challenge with PBS in the corneal graft (ipsilateral) eye and nothing in the contralateral eye. Graft recipients in the study by Beauregard et al received repeated mock challenges with PBS in the ipsilateral eye and repeated SRW challenges in the contralateral eye. One possibility is that, as mice rub their eyes vigorously after challenge with SRW, inadvertent contralateral transfer of SRW occurs. This also raises the possibility that accelerated graft rejection in these models of allergic conjunctivitis may be due to the mechanical effects of eye rubbing alone.

There is evidence to support the idea that the increased rate of rejection in allergic disease is due to systemic rather than local factors. Niederkorn’s group has gone on to study the effects of allergic airway disease on experimental corneal transplantation and found that in the presence of allergic airways disease, the tempo of corneal allograft rejection is also increased(Niederkorn et

al., 2009). This suggests that both allergic conjunctivitis and allergic airway disease have a similar effect on corneal graft survival and suggests either that this effect may be due to common systemic effects of allergy (eg Th2-bias) rather than localised tissue inflammation or that this effect on corneal allograft survival is caused by allergen-induced mucosal inflammation at either site.
In this paper the animals were sensitized with srw in the same way as per experimental allergic conjunctivitis. Allergen challenge is delivered intra-nasally. One potential concern here is again the delivery of a potent allergen to the face of mice and the risk of spread of allergen to the eyes during face/ eye rubbing after allergen delivery. This paper contained no data regarding the effect of intranasal allergen challenge on the conjunctiva. However, no eosinophils were found in rejected grafts in animals with allergic airway disease which suggests to me that these animals did not have allergic conjunctivitis(Niederkorn et al., 2009). Cursiefen reports decreased survival of corneal grafts in humans with atopic dermatitis(Nguyen et al., 2008). These patients may have allergic conjunctivitis but in this study the rate of rejection was increased even for those patients without a *reported* history of allergic conjunctivitis suggesting that allergic conjunctivitis is not the important factor in the increased rate of graft rejection seen in these patients. This finding is consistent with the work of Niederkorns group who attribute the accelerated rate of rejection in sensitized animals to “atopy”.

It is important to consider what the animal models of allergic conjunctivitis represent. A single challenge with allergen in a sensitized animal induces a condition which I believe to be analogous to severe seasonal allergic conjunctivitis in humans. Repeated exposure to allergen induces a chronic condition which may be more analagous to perennial allergic conjunctivitis (personal communication from Masaharu Ohbayashi). But what do sensitized animals represent? After sensitization with high doses of allergen these animals exhibit some of the systemic immunological features seen in human atopy such as increased titres of IgE and increased Th2 responses to antigen. However

atopy is a complex condition the precise cause of which remains uncertain and it may be an oversimplification to say that sensitization with allergen produces atopy. We must therefore interpret these results for sensitized^{+}challenged^{-} animals with caution when attempting to translate them in to the human setting.

There is also evidence to support the idea that the local effects of allergy may influence the immune response to alloantigen. Some of this evidence is indirect. For example Ozaki et al found increased expression of MHC class II in the cornea in experimental allergic conjunctivitis(Ozaki et al., 2004). In our laboratory we have reported increases in the number and alterations in the phenotype of conjunctival dendritic cells in response to allergen challenge(Ohbayashi et al., 2007). These findings raise the possibility that alloantigen recognition in the afferent limb may be enhanced in Sens^{+} Chall^{+} recipients of allografts.

De Vries et al have shown that allograft inflammation mediated by mast cell degranulation breaks peripheral tolerance of alloantigen(de Vries et al., 2009). This finding is highly relevant to our model as it has been shown that IgE-mediated mast cell degranulation is a key step in the mouse model of allergic conjunctivitis(Fukuda et al., 2009). De Vries showed that local mast cell degranulation induced systemic changes which caused breakdown of tolerance not only at the site of mast cell degranulation but also at distal tolerised allografts. They report a decrease in the number and function of Tregs and suggest that this may be the mechanism by which mast cell degranulation breaks down graft tolerance.

The idea of local degranulation of mast cells affecting allografts at other sites is very interesting because it could explain the discrepancy between my findings and those of Niederkorns group in relation to the fate of allografts in sensitized^{+}challenged^{-} animals.

	Local allergic inflammation	Distal allergic inflammation	Accelerated rejection	Eosinophils
Flynn	Y	N	Y	Y
	N	N	N	N
Beauregard	Y	Y	Y	Y
	N	Y (conj)	Y	N
	N	N	N	N
Niederkorn	N	Y (airway)	Y	N
	N	N	N	N

In all cases allografts in animals with mast cell degranulation either locally to the graft or distally are rejected at an increased tempo. In animals with neither local nor distal mast cell degranulation graft rejection is not accelerated.
However there are at least two reasons to doubt that mast cell degranulation is the missing link in these discordant data. Firstly, De Vries showed that mast cell degranulation in an isograft did not induce rejection of a tolerised distal allograft suggesting that widespread breakdown in tolerance is only caused by mast cell degranulation in allografted tissue and not autologous tissue. Secondly, DeVries' findings relate to tolerised grafts where Tregs have had time to develop and there is no evidence that these apply to newly transplanted grafts.

3.5.4. The effect of allergy on chemokine expression in corneal allograft rejection

To try to shed more light on the reason(s) for the presence of eosinophils in the graft during rejection an experiment was performed to determine chemokine expression during graft rejection. The hypothesis in this experiment was that greater expression of chemokines chemotactic for eosinophils (Eotaxin, RANTES) would be seen in allergic allografts than in naïve recipients. This would support the theory that eosinophils are actively recruited to the graft during acute rejection.

As far as eotaxin was concerned there there was no obvious difference and RANTES expression was *diminished* in allergic recipients compared to naïve. There are at least 2 explanations/ interpretations for/of these results. The first is that these results represent an accurate representation of differential chemokine expression between naïve and allergic recipients of allografts. In this case the results would not support the theory that eosinophils are actively actively recruited to the graft during acute rejection in allergic recipients. The other explanation is that these chemokine data represent 2 distinct snapshots of 2 distinct dynamic processes. Following transplantation, chemokines rise, plateau and fall at highly individual rates. These rates are different for each chemokine but are also influenced by the mileu of the graft. e.g. in the “high-risk” model chemokine expression of several chemokines are elevated in the early post-operative period compared to normal-risk grafts(Yamagami et al., 2005b). Early chemokine expression usually relates to innate immune cells infiltrating the wound with later chemokine expression correlating with effector cell influx. Because eosinophils made up part of the effector cell infiltrate in allergic recipients I was primarily interested in eosinophils and their chemokines around the time of rejection. Chemokine expression was measured at day 12 as it was one day before rejection was first seen in allergic recipients of allografts. In the naïve recipients chemokine expressin was also measure at day 12 (although in this group first rejection was not seen until day 15). The increased expression of lymphotactin, RANTES and IP-10 in naïve allografts compared with naïve isografts was consistent with previous reports of chemokine expression post corneal allotransplantaion(Yamagami et al., 1999,Pillai et al., 2008a). RANTES is produced by a wide variety of cell types and is a particulary pleiotropic chemokine with affinity for the receptors CCR1, CCR3 and CCR5(Levy, 2009). In experimental cardiac transplantation, gene knockout of either the CCR1 or CCR5 receptors improves graft survival(Gao et al., 2000,Gao et al., 2001). In experimental corneal transplantation gene knockout of CCR1 improves graft survival but knockout of CCR5 does not(Hamrah et al., 2007). IP-10 is produced by endothelium, fibroblasts and monocytes in response to IFN-γ. It has affinity

for the CXCR3 receptor on CD4 cells. There are conflicting reports regarding the benefit of targeting IP-10 and/or its receptor on graft survival in experimental cardiac transplantation. There has been one report of a study of experimental corneal transplantation in recipients with knockout of the IP-10 or CXCR3 genes, neither of which improved graft survival(Hamrah et al., 2007). Lymphotactin is produced by activated CD8 cells and has affinity for the CXCR1 receptor on T cells and NK cells(Hedrick and Zlotnik, 1998). I could find no reports of experiments where either lymphotactin or its receptor was targeted in studies of transplantation.

It is interesting that there was no obvious difference in chemokine expression between allergic recipients of allografts and allergic recipients of isografts. One possibility is that I missed a peak in chemokine expression in the allergic allograft group (either before or after day 12). My reasons for choosing this timepoint have been outlined in the methods section 3.4.2 of this chapter. I think that the most important thing I learned from this particular experiment was that, for comparative studies of post-operative chemokine expression, measurements at multiple timepoints are more useful than measurements at a single timepoint.

This technique has been used by many investigators to quantify the expression of RNA in ocular tissues(Yamagami et al., 1999,Ohta et al., 2000). In the image sent to me by my collaborators in Japan the film appears to have been slightly overexposed particularly in the lower part (Figure 3.12) which results in less clear-cut peaks and troughs in the right-hand part of the output (Figure 2.18; C) which made the intensity of the GADPH band, in particular, more difficult to measure. I tried unsuccessfully to reduce the background intensity using the "Subtract Background" function on ImageJ. I concluded that with the quality of film available to me this method was only semi-quantitative. I also realised that using this method I could not compare the chemokine expression within each sample as the relative background intensity increased further down each lane but I could compare the difference in intensity between lanes of each individual chemokine. I also realised that I was less likely to identify differences in

chemokine expression for the 3 chemokines at the lower (more saturated) part of the gel (MCP-1, TCA-3, Eotaxin) than those at the top (less saturated).

3.5.5. Chapter summary

The work presented in this chapter has demonstrated that:

- Allergic conjunctivitis at the time of corneal transplantation significantly increases the tempo of allograft rejection.
- Local conjunctival inflammation appears to be more important than systemic effects of sensitization to allergen in causing this change in the tempo of rejection.
- That graft and anterior uveal infiltration by eosinophils is seen during graft rejection in corneal graft recipients with allergic conjunctivitis.
- At a specific timepoint close to the first onset of rejection no marked increase in expression of the eosinophil-chemotactic chemokines RANTES and Eotaxin was seen in corneal recipients with allergic conjunctivitis compared with naïve recipients.

4. CHAPTER 4: The effect of allergic conjunctivitis on the immune response to allogeneic donor cornea: Innate immune cells, lymphangiogensis and the counter-effects of topical dexamethasone

4.1. INTRODUCTION

The results described in chapter 3 demonstrated that perioperative ipsilateral allergic conjunctivitis accelerated corneal allograft rejection and that this accelerated rate of rejection was associated with changes in the composition of the graft-infiltrating cell population during allorejection. Infiltration of the graft during allogeneic rejection is the end phase of a process which begins immediately after transplantation. The data described in chapter 3 suggested that local conjunctival inflammation is more important in accelerating rejection than systemic changes such as Th2 bias. Research from other laboratories has shown that the late phase reaction in allergic conjunctivitis peaks at 4-24 hours after allergen challenge(Choi and Bielory, 2008) and lasts 48-72 hours(Li et al., 1996) . With these data in mind, the following broad hypothesis was formulated: early cellular events in the cornea, which contribute to the process of rejection, may be modified in the setting of allergic conjunctivitis. A corollary of this hypothesis was that local treatment of allergic conjunctivitis would reverse or mitigate the modifications in the cellular events in the cornea and improve graft and reverse of mitigate the acceleration of graft rejection seen in the setting of allergic conjunctivitis.

Early cellular events following corneal transplantation are known to involve infiltration of innate immune cells such as macrophages and neutrophils(Kuffova et al., 2001). Normal cornea contains no blood or lymphatic vessels and this contributes to its immune privileged status. Pre-existing blood vessels in the cornea are a recognised risk factor for rejection but new blood vessels may also grow in to an avascular cornea after transplantation and these also increase the risk of rejection(Chung et al., 2009,Cursiefen et al., 2004b). In the other well-characterised model of high rejection risk corneal transplantation, in which donor cornea is transplanted into a vascularised recipient corneal bed, early infiltration of the graft with neutrophils and macrophages is significantly increased compared with normal/ low rejection risk grafts although the precise role and degree of influence of these cells on sensitisation and rejection dynamics

remains unclear(Yamagami et al., 2005b). Vascularised recipient cornea also contains lymphatic vessels which facilitate sensitisation of the host to alloantigen. Data presented in Chapter 3 demonstrated that the sensitisation process did not induce corneal lymphatics (i.e. there were no pre-existing lymphatics at the time of transplantation). However it is now known that corneal lymphatics grow in to the graft host interface after transplantation(Cursiefen et al., 2004a) . Corneal blood vessels are easy to study as they are visible in vivo. It is now recognised that corneal angiogenesis is accompanied by corneal lymphangiogenesis. Corneal lymphangiogenesis has been shown to be induced by innate / alloantigen-independent inflammation in the cornea (Cursiefen et al., 2004b) but provides a conduit for egress of APCs which sensitise the host to alloantigen. With the discovery of new markers for lymphatic endothelium, there has been renewed interest in corneal lymphangiogenesis not only for its role in corneal transplant immunology but also as a model for studies of lymphangiogenesis in relation to cancer metastases(Achen and Stacker, 2008).

New markers for lymphatic endothelial cells have been used by several groups to confirm the presence of lymphatic vessels in normal mouse conjunctiva. A population of individual non-vascular cells in the conjunctiva have been shown to express markers of lymphatic endothelium and it has been proposed that these cells contribute to the ingrowth of new corneal lymphatic vessels in response to corneal inflammation(Chen et al., 2005,Xu et al., 2007).

A more specific hypothesis, therefore, was that allergic conjunctivitis may modify both the number of $CD11b^{+}$ cells (innate immune cells) and the ingrowth of new lymphatic vessels entering the host cornea after transplantation.

4.2. THE EFFECT OF TREATMENT OF ALLERGIC CONJUNCTIVITIS ON GRAFT SURVIVAL

4.2.1. Research question

What is the effect of short term post-operative olopatadine and dexamethsone 0.1% treatment on corneal allograft survival in the setting of perioperative allergic conjunctivitis?

4.2.2. Methods/ Experimental design

The techniques of induction of allergic conjunctivitis and corneal transplantation were used in this experiment. These techniques have been described in detail in chapter 2 and modified slightly as described below.

The experimental design was as shown in Figure 4.1. Animals were challenged with short ragweed pollen immediately after completing suturing of the corneal allograft. After 5 minutes a drop of PBS/ dexamethasone 0.1% (Maxidex, Alcon, Hunnenberg, Switzerland) or olopatadine (Opatanol, Alcon,) or dexamethasone 0.1% & olopatadine was instilled in the grafted eye. A blephorraphy was performed and this was opened the next day and drops were instilled in the grafted eye twice daily for one week. Thereafter graft clarity was assessed three times per week as described in chapter 2.

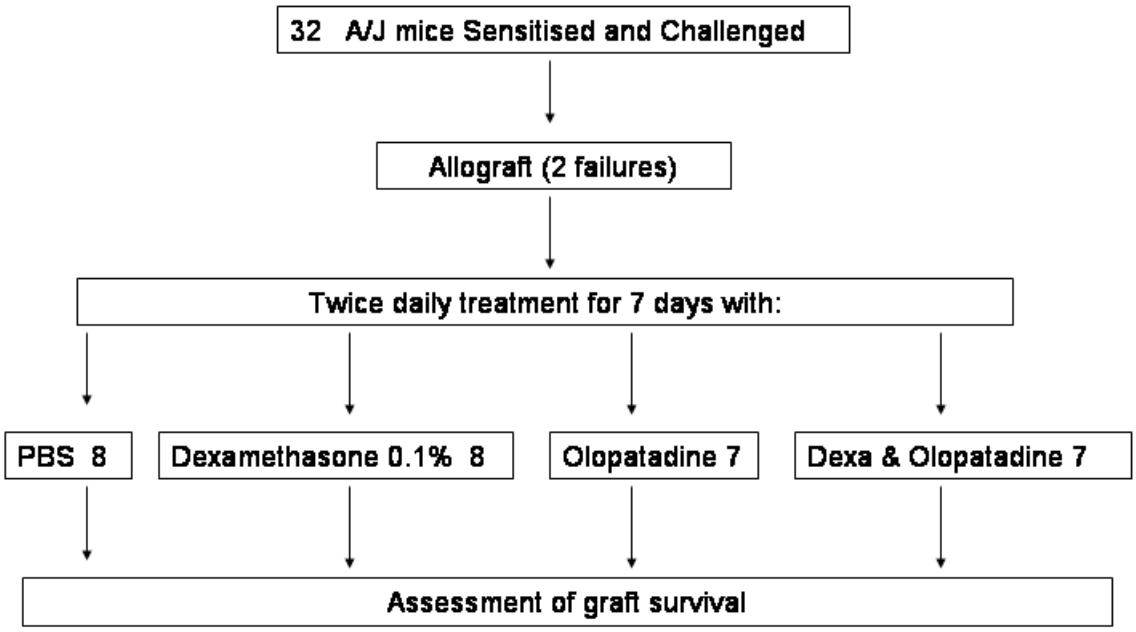


Figure 4.1 Experimental design part 1

4.2.3. Results

Median survival in PBS-treated animals was 21 days. In comparison, survival in dexamethasone treated animals was significantly prolonged (MST 30 days; p=0.008; Figure 4.2). Survival in animals treated with both dexamethasone 0.1% and olopatadine was also significantly prolonged (MST 55 days; p=0.003). However survival in animals treated by olopatadine alone (MST 24 days) was not significantly different from that in animals treated with PBS (MST 21 days; p= 0.547). Furthermore although the difference in MST between those treated with dexamethasone alone (30 days) and those treated with dexamethsone and olopatadine (55 days) appeared quite large, this difference did not reach statistical significance (p= 0.517). Median graft survival times in each group are shown in Table 4.1. P values for pairwise comparison of allograft survival between treatment groups using the Log Rank test are shown in table 4.2. These data suggest that the effect of perioperative allergic conjunctivitis on corneal allograft survival may be mitigated by twice daily perioperative treatment with dexamethsone 0.1% but not olopatadine.

Donor	Recipient	Treatment for 7 days	MST
C57BL6	A/J with allergic conjunctivitis	PBS bd	21
C57BL6	A/J with allergic conjunctivitis	Dexamethsone 0.1% bd	30
C57BL6	A/J with allergic conjunctivitis	Olopatadine bd	24
C57BL6	A/J with allergic conjunctivitis	Dexamethsone 0.1% bd & Olopatadine bd	55

Table 4.1 Median survival times of corneal allografts in each treatment group

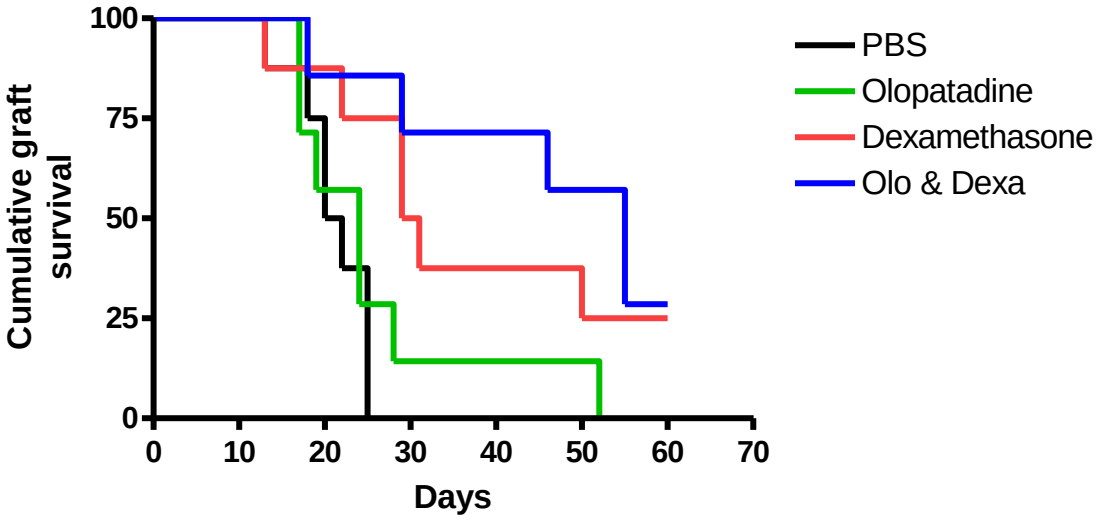


Figure 4.2 Effect of perioperative topical anti-inflammatory treatment on corneal graft survival in recipients with allergic conjunctivitis

Treatment with twice-daily topical dexamethasone 0.1% drops for one week after surgery (red curve) significantly prolonged corneal allograft survival in eyes with perioperative allergic conjunctivitis compared to treatment with twice daily PBS drops (black curve).

	PBS	Dexamethasone	Olopatadine	Dexametathasone & Olopatadine
PBS		0.008	0.547	0.003
Dexamethasone	0.008		0.101	0.517
Olopatadine	0.547	0.101		0.011
Dexametathasone & Olopatadine	0.003	0.517	0.011	

Table 4.2 P values for pairwise comparison of allograft survival between treatment groups using the Log Rank test.

4.3. THE EFFECT OF ALLERGIC CONJUNCTIVITIS ON CORNEA-INFILTRATING CD11b$^+$ CELLS

4.3.1. Research question

What is the effect of perioperative allergic conjunctivitis on the number of host cornea-infiltrating CD11b$^+$ cells in response to corneal allotransplantation?

4.3.2. Methods/ Experimental design

The techniques of induction of allergic conjunctivitis and corneal transplantation were used in this experiment. These techniques have been described in detail in chapter 2. The experimental design for this experiment was as described in Figure 4.3. Eyes were eviscerated at days 2 and 6 and fluorescent immunohistochemical staining was performed as described in chapter 2 using the following antibodies:

FITC-conjugated anti-F4/80
PE-conjugated anti-CD11b
Anti-LYVE-1
Anti-Gr1
Alexa 488-conjugated Donkey anti-Rat IgG

Chapter 2 contains a detailed description of these antibodies and optimised concentrations and conditions for their use in immunohistochemistry.

Single colour images of cross sections of the host cornea, the graft-host junction and the centre of the graft were captured on a fluorescence microscope (Olympus). For 2-colour staining, images were captured using a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

Positive-staining cells in a x 400 field of the host cornea were counted by a masked observer. Cells were counted in 3 sections per rejected graft. At least 4 eyes per timepoint were examined in each group. Mean number of infiltrating cells were calculated.

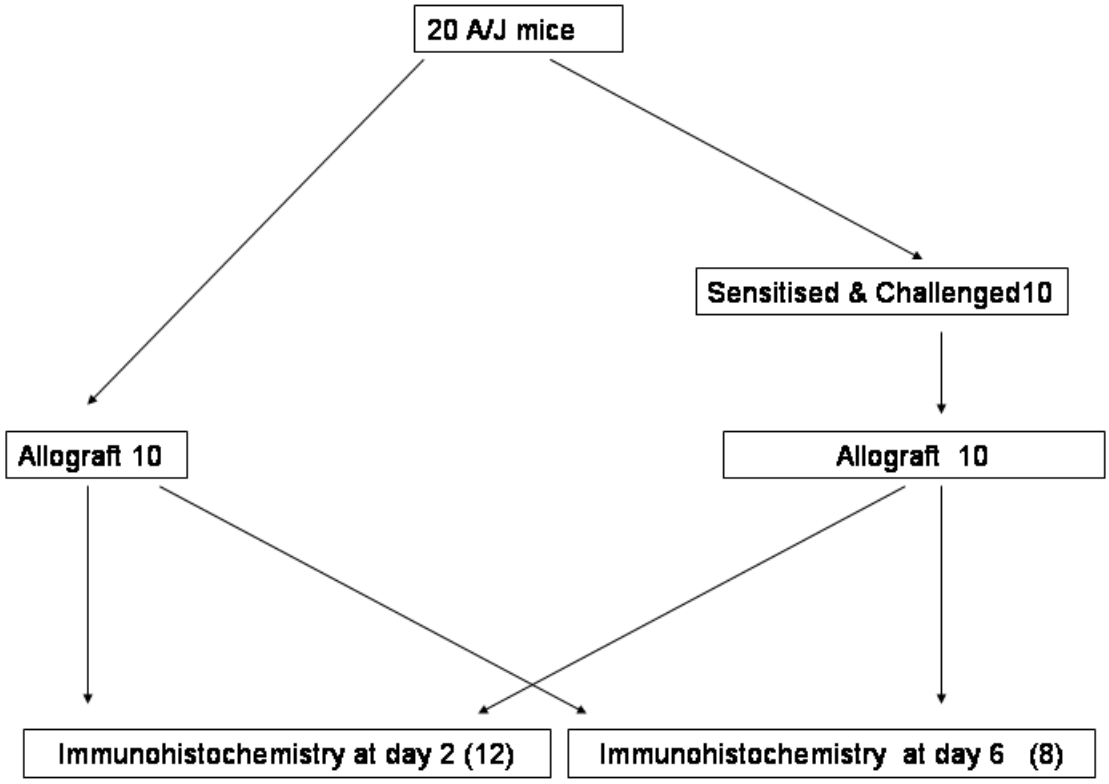


Figure 4.3 Experimental design part 2

4.3.3. Results

Aggregates of $CD11b^+$ cells were seen at the limbus of both naïve and allergic recipients of allografts at 48 hours (Figure 4.4;B) . At this timepoint inflammatory cell infiltration of the cornea was greater in the host than the graft (Figure 4.4; C). Significantly more $CD11b^+$ cells were seen in the host cornea at 48 hours post

corneal transplantation in allergic eyes than in naïve eyes (84.46 v 38.14; p= 0.029; Figure 4.5). Two morphologically and immunohistochemically distinct subsets of $CD11b^+$ cells were seen in the host cornea: ovoid $CD11b^+$ $Gr\text{-}1^+$ cells (neutrophils)and more elongated $CD11b^+$ $F4/80^+$ cells (macrophages)(Figure 4.5; C, D). Relatively few $CD11b^+$ cells were seen in the centre of the graft at 48 hours post-transplantation and there was no significant difference in the numbers of $CD11b^+$ cells in the centre of the graft at this timepoint between naïve (8.13 +/- 3.85, mean=+/- SE) and allergic (14.53 +/- 7.74) graft recipients (p= 0.48).

By day 6 post-transplantation large numbers of $CD11b^+$ cells were seen in the graft as well as the host cornea (Figure 4.4; E). At day 6 post-transplantation the host cornea in allergic recipients contained slightly more $CD11b^+$ cells than naïve recipients but this difference was not statistically significant (Figure 4.5; E).

Linear and circular $LYVE\text{-}1^+$ structures could be seen in the host cornea of both naïve and allergic recipients (Figure 4.6). The circular structure was seen at the limbus and represents a large annular lymphatic vessel. The linear structures represent smaller lymphatic vessels growing into the host cornea. These structures were $LYVE\text{-}1^+CD11b^-$ and appeared to grow in the superficial stroma/ subepithelial plane (Figure 4.6). A small number of the $CD11b^+$ cells entering the cornea following corneal transplantation also stained positive for LYVE-1 (Figure 4.6; C).

It would have been possible to measure the length of linear ingrowth of these $LYVE\text{-}1^+$ structures as a way to compare lymphatic ingrowth between naïve and allergic recipients of corneal allografts. For reasons discussed in Chapter 2 a new experiment was planned which would use corneal wholemount staining to assess lymphatic ingrowth.

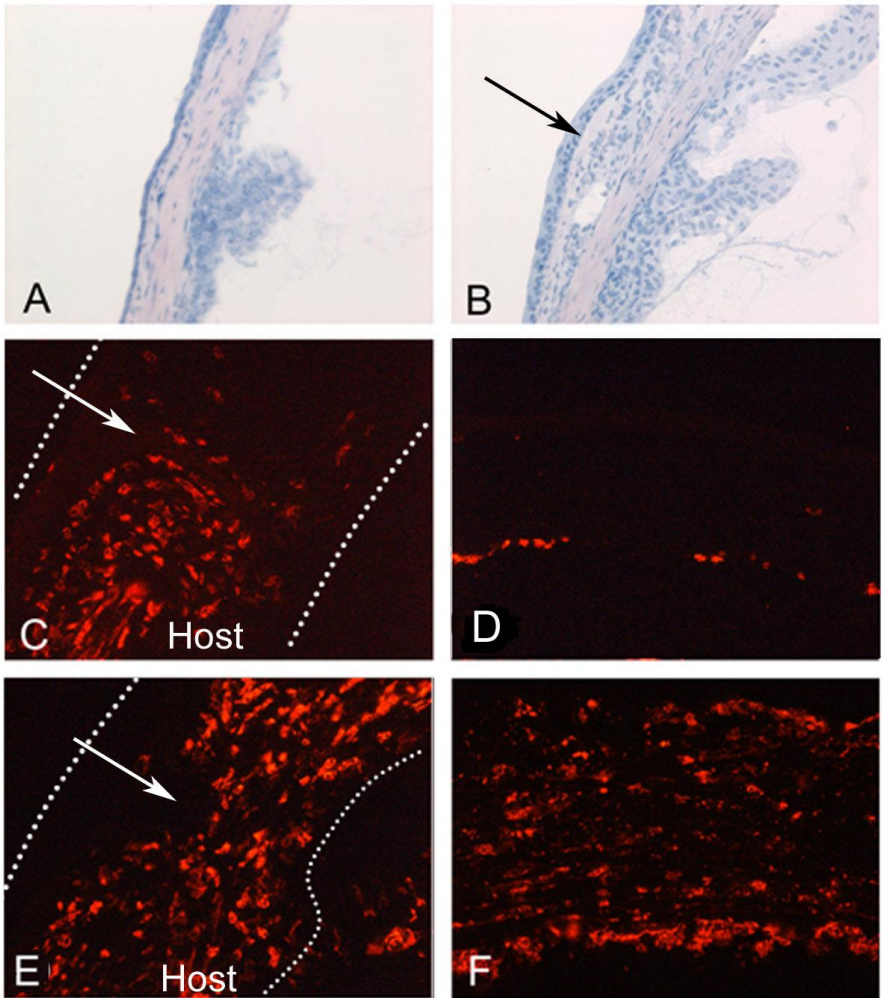


Figure 4.4 Entry of CD11b+ cells to the cornea after corneal transplantation

(A) A photomicrograph of part of a haematoxylin-stained section of a normal mouse eye showing the limbal conjunctiva. (B) A haematoxylin-stained section showing the corresponding part of a mouse eye 2 days post corneal transplantation. Large numbers of cells have aggregated in the limbal conjunctiva forming a “hump” in cross-section (black arrow). (C) A section showing the graft-host junction (white arrow) at 2 days post- corneal transplantation. Following corneal transplantation $CD11b^+$ cells (red) enter the host cornea presumably via the limbal conjunctiva. Large numbers of these cells are seen in the host cornea but few have crossed the graft-host junction and entered the graft stroma. (D) A section showing the graft at 2 days post-corneal transplantation. A few $CD11b^+$ cells are seen in close proximity to the graft endothelium but relatively few cells have infiltrated the graft at this point. (E) A section showing the graft-host junction (white arrow) at 6 days post- corneal transplantation. Large numbers of $CD11b^+$ cells (red) are now seen in both the host cornea and the graft. (F) A section showing the graft containing many $CD11b^+$ cells (red) at 6 days post-corneal transplantation.

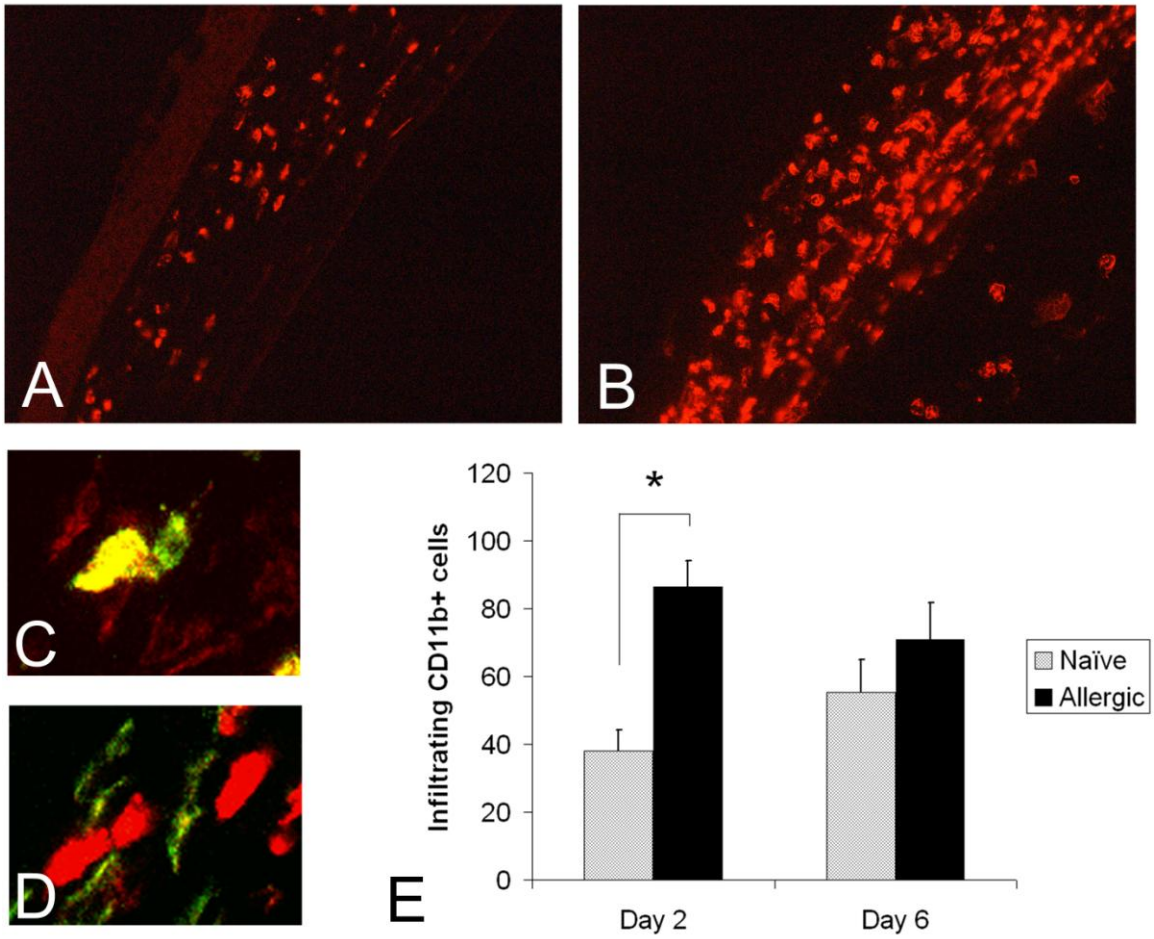


Figure 4.5 CD11b+ cells in host cornea

At day 2 significantly greater numbers of $CD11b^+$ cells were seen in the host cornea of allergic (B; CD11b=red) allograft recipients than naïve (A; CD11b=red) recipients (E; * p=0.029). There was no significant difference at day 6. Two morphologically and immunohistochemically distinct subsets of $CD11b^+$ cells were seen in the host cornea: ovoid $CD11b^+$ $Gr\text{-}1^+$ cells (CD11b=red, Gr-1=green; C) and cigar-shaped CD11b+ F4/80+ cells (CD11b=red; F4/80=green; D)

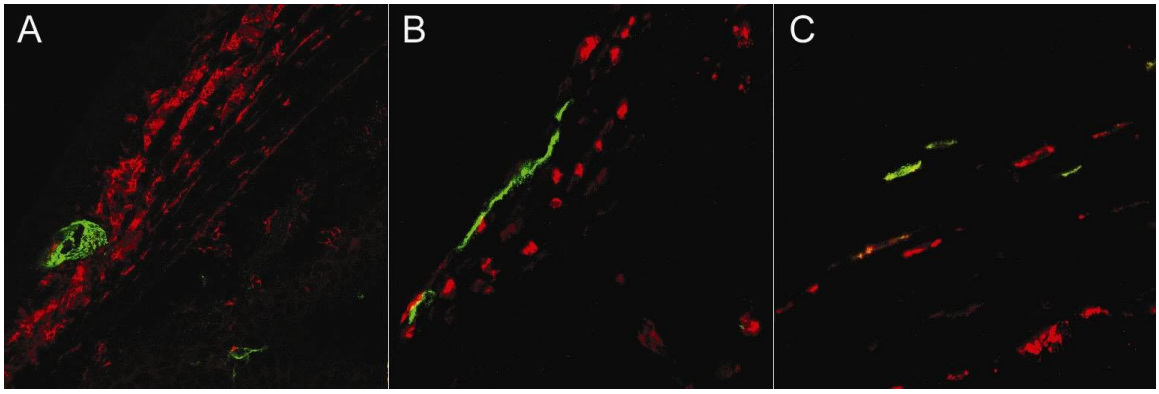


Figure 4.6 LYVE-1 expression in host cornea.

Accompanying the influx of $CD11b^+$ cells (red) into host cornea of both naïve and allergic recipients of corneal allograft there were circular (A) and linear (B) structures which stained positive for LYVE-1(green). Only occasional $CD11b^+$ $LYVE\text{-}1^+$ double positive cells (yellow) were found amongst the infiltrating cells (C).

4.4. THE EFFECT OF ALLERGIC CONJUNCTIVITIS ON CORNEAL LYMPHANGIOGENESIS

4.4.1. Research questions

What is the effect of perioperative allergic conjunctivitis on corneal lymphangiogenesis in response to corneal allotransplantation?

Is any observed effect of perioperative allergic conjunctivitis on corneal lymphangiogenesis mitigated by use of topical g Dexamethasone 0.1%?

4.4.2. Methods/ Experimental design

The techniques of induction of allergic conjunctivitis and corneal transplantation were used in this experiment. These techniques have been described in detail in chapter 2. The experimental design for this experiment was as described in Figure 4.7. The technique for staining and imaging lymphatic vessels ingrowth in whole corneal wholemounts is described in detail in Chapter 2. Wholemounts were imaged by fluorescent microscopy (Olympus). An image of each quadrant was captured with original magnification of x100. Radial ingrowth of LYVE-1 $^{+}$ vessels from the limbus was measured in each quadrant by a masked observer using image analysis software (Soft Imaging System GnbH, Munster, Germany). First the system was calibrated by photographing a 1mm scale at the same magnification (x100). Using a freehand drawing tool on the software a line was traced manually over each lymphatic vessel and its branches. The software automatically calculated the length of each line and these values were recorded and the combined total length of lymphatic vessels in each quadrant was calculated. The totals for each quadrant were added to give a measure of lymphatic ingrowth for each cornea. At least 4 corneas were analysed per group per timepoint. Mean values were calculated.

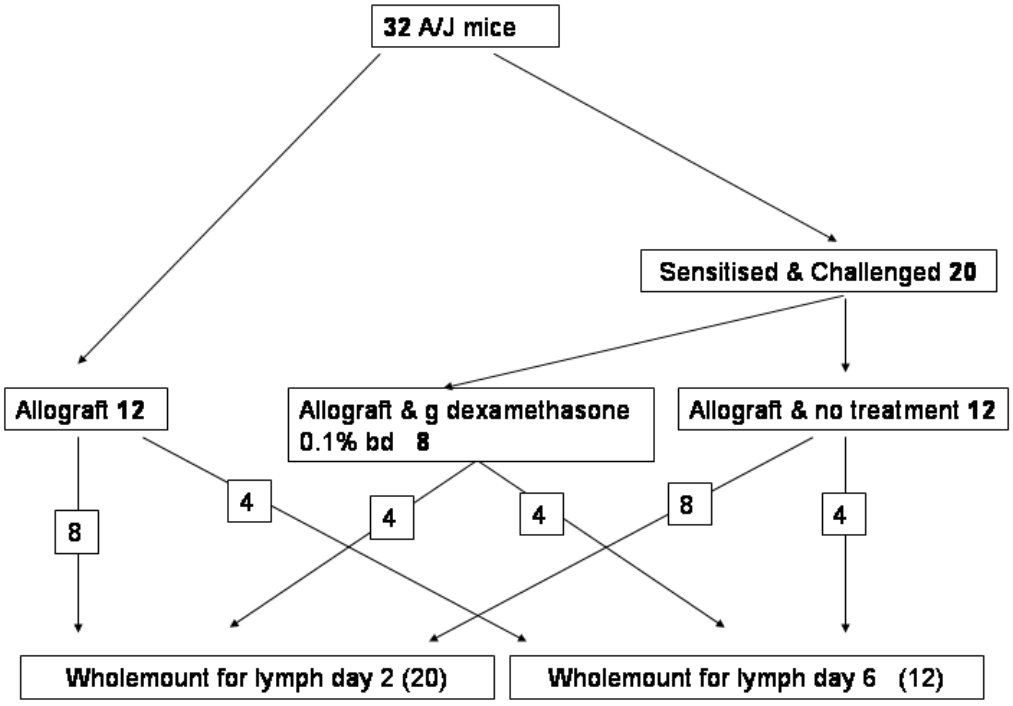


Figure 4.7 Experimental design part 3

4.4.3. Results

At day 2 post-transplantation significantly more LYVE-1^+ radial vessel ingrowth was detected in allergic recipients of allografts than in naïve recipients (cumulative length 2564 μm v 1189 μm; p=0.014; Figure 4.8). At day 6 post-transplantation the lymphatic ingrowth approached the graft-host interface and there was no significant difference between allergic and naïve recipients of corneal grafts (cumulative length of 12908μm v 13527μm). No lymphatic ingrowth beyond the graft-host interface was seen at this timepoint.

At days 2 and 6 post transplantation radial ingrowth of LYVE-1^+ vessels was significantly reduced in dexamethasone-treated allergic recipients of allograft compared with untreated recipients (Figure 4.9; cumulative length at day 2 of 2564μm v 1141μm; p= 0.029; cumulative length at day 6 of 12908μm v 3387μm; p= 0.009)

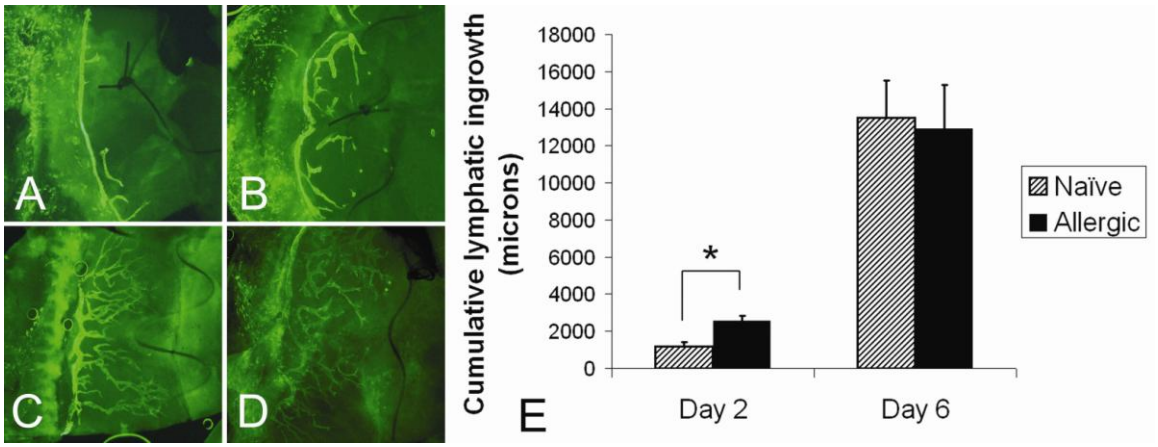


Figure 4.8 Corneal lymphangiogenesis after corneal allotransplantation

Panels A-D show representative images of corneal wholemounts stained with anti LYVE-1 in naïve (A, C) and allergic (B, D) recipients of corneal transplants at day 2 (A, B) and day 6 (C, D) post-transplatation. Panel E is a histogram describing the mean cumulative lymphatic ingrowth (error bars show SEM). At day 2 post-transplantation significantly more LYVE-1^{+} radial vessel ingrowth (green) was detected in allergic recipients (B) of allografts than in naïve recipients(A, E; * p=0.014) There was no statistically significant difference in lymphatic ingrowth at day 6 between naïve (C) and allergic (D) graft recipients.

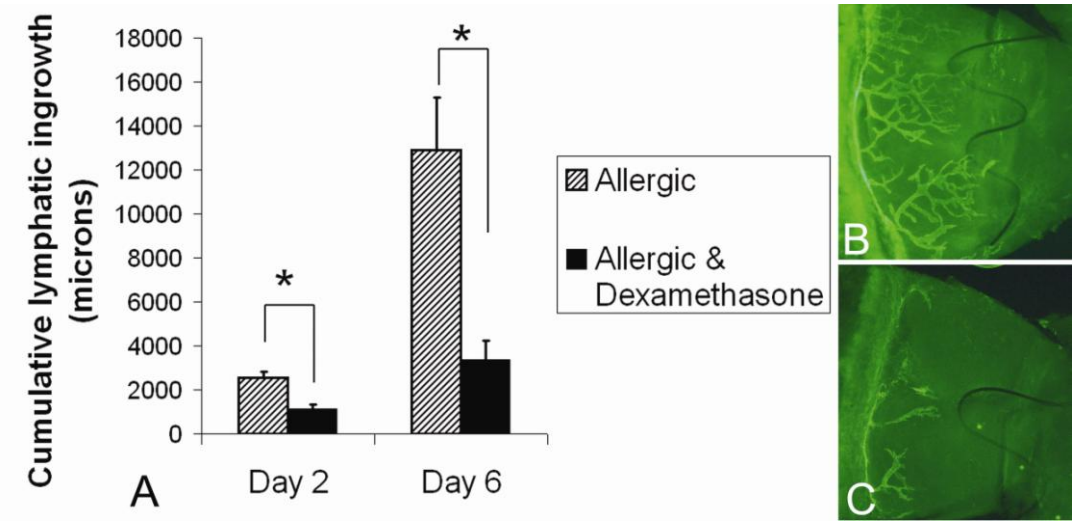


Figure 4.9 Effect of Treatment on corneal lymphangiogenesis

Panel A is a histogram describing the mean cumulative lymphatic ingrowth (error bars show SEM). Panels B and C are representative pictures of corneal wholemounts stained with anti-LYVE-1 at days 6 post corneal transplantation in an allergic recipient (B) and an allergic recipient treated with 6 days of twice-daily dexamethasone 0.1% (C). Treatment with twice-daily topical dexamethasone 0.1% significantly inhibits corneal lymphangiogeneis at days 2 ($p=0.029$) and 6 ($p=0.009$) in allograft recipients with allergic conjunctivitis (A; *= $p<0.05$). Extensive LYVE-1^+ vascular ingrowth can be seen on the wholemount of a cornea at 6 days post-transplantation in an untreated recipient with perioperative allergic conjunctivitis (B). By comparison, relatively little ingrowth is seen at day 6 in the recipient treated with twice daily dexamethasone 0.1% drops (C).

4.5. DISCUSSION

4.5.1. CD11b$^+$ cells in the host cornea

The finding of increased numbers of CD11b$^+$ cells in host cornea at day 2 post – transplantation in allergic recipients of corneal grafts is a novel finding. CD11b is a functional molecule which together with CD18, forms part of the integrin Mac-1 which regulates leukocyte adhesion and migration(Solovjov et al., 2005). Mac-1 is expressed on macrophages and several authors appear to use CD11b as a marker of macrophages(Chen et al., 2005,Maruyama et al., 2005). However Mac-1 is also expressed on neutrophils. Accordingly the CD11b$^+$ cells in the cornea in my experiments were seen to stain not only with antibodies against markers macrophages (F4/80) but also against those for neutrophils (Gr-1). These cells represent the early innate response to corneal injury. It is also possible that the population of CD11b$^+$Gr-1$^+$ in the cornea following transplantation represents myeloid suppressor cells. If so, the net effect of the increased numbers of these cells seen in the setting of allergic conjunctivitis may be to cause a reduction in corneal inflammation.

In the vascularisation high-risk model of corneal transplantation, early infiltration with neutrophils and macrophages was also increased(Yamagami et al., 2005b). This high-risk model is characterized by the presence blood and lymphatic vessels in the host cornea prior to transplantation. Vascularisation of the cornea provides ready access for ingress and egress of immune cells. Because a normal cornea contains few/no blood vessels, inflammatory cells enter the corneal stroma, when required, via the limbal conjunctiva. In the allergic conjunctivitis model the conjunctiva was seen to be heavily infiltrated with CD11b$^+$ cells at 48 hours post transplantation(Ohbayashi et al., 2007,Fukushima et al., 2009). This conjunctival inflammation appears to facilitate or cause the entry to the cornea of increased numbers of inflammatory cells.

My data suggest that at 2 days post-transplantation most of the inflammatory cells in the cornea are confined to the host. Relatively few cells were seen in the graft at this stage but this situation changes at day 6 when large numbers of $CD11b^+$ cells are seen in the centre of the graft. These data are not consistent with those of Kuffova who found a sustained peak of $CD11b^+$ cell infiltration of the graft between 6 hours and 6 days post-transplantation(Kuffova et al., 2001) . In my model there appears to be a lag-time for entry of $CD11b^+$ cells to the graft. Whether this is due to a relative barrier effect of the graft-host interface or simply due to the fact that the cells are moving in a centripetal direction and take more time to reach the centre is not clear.

4.5.2. Corneal lymphangiogenesis

There appears to be a strong relationship between infiltration of the cornea with $CD11b^+$ cells and the development of new lymphatic vessels(Maruyama et al., 2005). This has been attributed mostly to the macrophage cohort of $CD11b^+$ cells although the exact mechanism has not been established.
A population of $CD11b^+$ cells in the conjunctiva have been found to also stain for the lymphatic endothelial markers LYVE-1(Xu et al., 2007) and VEGFR-3(Hamrah et al., 2004). One theory that has been suggested is that these cells enter the cornea in response to inflammation and incorporate themselves into developing lymphatics. Dana found that the typical response to a corneal suture was a decrease in the numbers of conjunctival $LYVE\text{-}1^+$ cells and an increase in the numbers of corneal $LYVE\text{-}1^+$ cells suggesting that the conjunctival $LYVE\text{-}1^+$ cells enter the cornea and contribute to lymphangiogenesis(Chen et al., 2005). In our experiments we found that very few $CD11b^+$ cells infiltrating the cornea were $LYVE\text{-}1^+$. In addition many $CD11b^+$ cells were $Gr\text{-}1^+$ (neutrophils) and not $F4/80^+$ (macrophages). This suggests that only a proportion of the infiltrating cells have the capacity to contribute directly to lymphangiogenesis. Lymphatic vessels in the cornea and conjunctiva were $CD11b^-$ on immunohistochemistry

suggesting either that newly recruited $CD11b^+LYVE\text{-}1^+$ cells did not contribute directly to the new lymphatic endothelium or that these cells lose the CD11b marker once they incorporate into the new lymphatic endothelium. In a study of de novo lymphangiogenesis in human renal transplantation Kerjaschki et al also found that endothelial progenitor cells did not retain the CD11b marker once incorporated into new lymphatics(Kerjaschki et al., 2006). Maruyama has described $LYVE\text{-}1^+CD11b^+$ double positive vessels in the cornea on wholemount(Maruyama et al., 2005). We did not perform double staining in corneal wholemounts but it is possible that there is an inconsistency in staining between the techniques of immunohistochemistry and corneal wholemount. Another theory regarding the role of $CD11b^+$ macrophages in corneal lymphangiogenesis is that they release VEGF-C, a potent pro-lymphangiogenic cytokine which is a ligand for VEGFR3(Kerjaschki, 2005).

The role of neutrophils (which are also $CD11b^+$) in this process has not been explored. Cursiefen et al report that neutrophils are the dominant $CD11b^+$ cells in cornea at one week after corneal suture placement(Cursiefen et al., 2004b). A later paper by the same group describes the use of clotidronate liposomes to inhibit $CD11b^+$ influx to the cornea after transplantation and found that in this setting lymphangiogenesis was inhibited(Maruyama et al., 2005). From the data presented in their papers, clotidronate liposomes appeared to inhibit influx of all $CD11b^+$ cells to the cornea and to inhibit corneal lymphangiogenesis but the differential roles of macrophages and neutrophils were not explored.

Shimizu has studied the role of the Mac-1 molecule in cardiac transplantation and found that, in a mouse model, cardiac transplantation to Mac-1 KO recipients improved graft survival as compared to WT recipients. To investigate whether this effect was due to impaired macrophage function or impaired neutrophil function, Mac-1 KO graft recipients were reconstituted with WT macrophages and WT neutophils. Adoptive transfer of WT macrophages reduced survival while adoptive transfer of WT neutrophils did not affect survival suggesting that any

functional role of Mac-1/ CD11b in cardiac transplantation is due to its presence on macrophages but not on neutrophils(Shimizu et al., 2008). On other hand there are reports which support a role for neutrophils in rejection of cardiac allografts. Morita depleted neutrophils in cardiac graft recipients using the anti-Ly6G mAb, RB6.8C5 and found that this improved graft survival(Morita et al., 2001).

If inhibiting CD11b+ influx inhibits lymphangiogenesis, does increasing $CD11b^+$ influx accelerate lymphangiogenesis? Based on my observations described above, the answer appears to be yes but only very slightly. The difference between allergic and naïve eyes was marginal (but statistically significant) at day 2 and there was no difference at all at day 6 by which time lymphangiogenesis had reached the graft-host interface in both groups. Although at day 6 $CD11b^+$ cells had entered the graft no lymphatic vessels were evident in the graft at this time suggesting either a) that $CD11b^+$ cell infiltration is necessary but not sufficient for corneal lymphangiogenesis b) that the wound at the graft host interface provides a barrier to lymphatic ingrowth or c) that there is a lag between $CD11b^+$ cell influx and lymphangiogenesis and that lymphatic vessels may have been observed in the graft in a group at a later timepoint if such a group had been included in the experimental design.

4.5.3. Inhibition of corneal lymphangiogenesis with topical corticosteroid

Investigators have reported inhibition of corneal lymphangiogenesis using inhibitors of VEGF-A or VEGFR-3 with associated improvements in graft survival (Chen et al., 2004,Bachmann et al., 2008). Post-operative topical corticosteroids are currently used on all human corneal graft recipients but until now we have known little about their effect on corneal lymphangiogenesis. Collin described inhibition of limbal lymphatic growth by topical corticosteroid over 20 years ago(Boneham and Collin, 1995) and we have confirmed this finding in the setting of corneal allotransplantation. There is experimental evidence that topical

corticosteroids inhibit influx of $CD11b^+$ innate immune cells to the cornea and corneal haemangiogenesis(Basu et al., 1981,Nakao et al., 2007). Inhibition of innate immune cell infiltration of the host cornea and/ or inhibition of VEGF-A secretion by infiltrating cells may be the mechanisms by which topical corticosteroid inhibits lymphangiogenesis.

4.5.4. Effect of topical treatment on graft survival in recipients with allergic conjunctivitis

Short-term post-operative topical dexamethasone treatment improved long-term corneal allograft survival. The local effects of corticosteroids on the immune response are many and varied and it is implausible that inhibition of lymphangiogenesis is the sole reason for the improvement in graft survival seen. However, it is likely to be a contributory factor.

One question that has not been addressed, and which would help to decipher how much of the beneficial effects of dexamethasone 0.1% is due to the suppression of allergic conjunctivitis and how much is due to other effects of the treatment, is: what is the effect of topical dexamethasone 0.1% on the survival and post-operative lymphangiogenesis in naïve recipients of corneal allografts? I became aware of this weakness in my experimental design relatively late and was unable to revisit this question. However, other laboratories have demonstrated a beneficial effect of topical corticosteroid on survival time in low rejection risk small animal corneal transplantation (Zhang et al., 2000,Williams et al., 1987).

Short-term post-operative topical olopatadine treatment did not improve long-term corneal allograft survival. This may be interpreted in a number of ways. On one hand it may be interpreted as indicating that treatment of perioperative allergic conjunctivitis is not effective at improving corneal graft survival. Given that olopatadine has less far-ranging effects on immune function than dexamethasone and, as such, is a more specific therapy for allergic conjunctivitis

these data appear to support the proposal by Niederkorn that the accelerated rate of rejection in atopic mice is not related to local conjunctival inflammation. On the other hand, we must bear in mind the mechanism of actions of olopatadine which are mast cell stabilization and antihistamine effect. Mast cell degranulation and histamine release are key events in the early phase of allergic conjunctivitis(Fukuda et al., 2009). There is currently much interest in the role of mast cells in tolerance induction in acquired immune responses. DeVries recently showed that mast cell degranulation led to rejection of previously tolerised skin grafts and that this effect was mitigated by prior stabilization of mast cells(de, V et al., 2009). Although a mast-cell stabilizing drug (olopatadine) was used in this experiment it is probably not correct to say that the effect of mast cell stabilization on corneal graft survival in the setting of allergic conjunctivitis has been tested. Mast cell degranulation occurs rapidly, extensively and explosively in our model in response to a once-off exposure to allergen. Therefore subsequent treatment with olopatadine as in this study may be likened to “closing the barn door after the horse has bolted”. To test the effectiveness of a mast cell stabilser on allergic conjunctivitis and graft survival it may have been better to use an additional group who were treated before exposure to allergen as in DeVries’ study.

Our results suggest enhancement of lymphatic vessels running in a subepithelial plane in the cornea in response to allergen-induced conjunctival inflammation in the hours following corneal transplantation. It is possible that this may facilitate APC movement from the cornea to the draining lymph nodes and that this contributes to the accelerated rejection seen in the setting of allergic conjunctivitis. Whether these phenomena are caused specifically by conjunctival inflammation induced by allergen challenge and not by conjunctival inflammation of other causes is not known but I would think this unlikely. It is probable that conjunctival inflammation induced by any cause would have a similar effect but I did not test this hypothesis as I was unaware of an equally humane and reproducible method of inducing non-allergic conjunctivitis in mice. Survival data from such an experiment would certainly shed further light on the question of

whether local inflammation or systemic immune effects of allergy/atopy are responsible for the increased tempo of corneal graft rejection seen in recipients with allergic conjunctivitis.

4.5.5. Chapter summary

The work presented in this chapter has demonstrated the following:

- Short-term perioperative treatment with dexamethasone 0.1% drops mitigates the adverse effect of perioperative allergic conjunctivitis on corneal allograft survival.
- Corneal allograft recipients with perioperative allergic conjunctivitis have significantly greater numbers of host cornea-infiltrating $CD11b^{+}$ leukocytes at 2 days post transplantation than naïve recipients.
- Corneal allograft recipients with perioperative allergic conjunctivitis have slightly greater centripetal ingrowth of new lymphatic vessels at 2 days post transplantation than naïve recipients.
- The ingrowth of new corneal lymphatic vessels after corneal transplantation in animals with allergic conjunctivitis is significantly inhibited by treatment with dexamethasone 0.1% drops.

5. CHAPTER 5: Understanding the mouse model of corneal allograft rejection

5.1. INTRODUCTION

Most information on the sequence of events in rejecting corneas has been obtained from experimental animal models of corneal transplantation(Larkin, 1994). The descriptive analyses of events in unmodified corneal graft rejection have come from the inbred rat(Williams and Coster, 1985,Larkin et al., 1997b), mouse(Zhang et al., 1996,Yamagami et al., 1999) and rabbit(Rayner et al., 2000) models of transplantation. Corneal transplantation in small rodents is technically very difficult, but they have the important advantage over larger animals in that genetically identical inbred strains are available, which allows control for many experimental variables. Studies from these species have demonstrated a correlation between inflammatory infiltrates in aqueous humour samples from the anterior chamber, pathological sections of cornea and local lymph nodes. However a problem with descriptive studies, and to a greater extent in studies of experimental therapies in all mouse and rat graft transplantation models, is that rejection is diagnosed subjectively on the first post-operative examination day on which graft transparency is lost. This end-point can be difficult to determine and the subjectivity may hinder determination of graft survival. In contrast, the onset of rejection in rabbit allografts is indicated by endothelial or epithelial rejection lines.

During the experiments described in chapters 3 and 4 three specific problems for identifying graft rejection in mice were identified:

1. The loss of iris detail can be a subjective phenomenon.
2. Occasionally grafts opacify and linger at grades 1-2 for a relatively long period before reaching grade 3.
3. Occasionally grafts opacify in one part of the graft only so that part of the graft would be at Grade 0 and part would be at Grade 3. (Figure 5.1)

In addition the temporal relationship between loss of graft clarity and immune-mediated inflammation in the graft remains unclear. For example it was

sometimes found that grafts of opacity grade 4 would have relatively few graft-infiltrating cells as compared with those of opacity grade 3. This apparent anomaly may have implications for studies of effector cell mechanisms in graft destruction.

It would be a significant advance if thickness of the very thin cornea in rodents could be directly measured at sequential examinations following transplantation. In this way, it has been possible to perform longitudinal objective measurements of rabbit graft thickness using a clinical pachymeter, modified for use in rabbit in which central thickness is 330μ(Rayner et al., 2000).

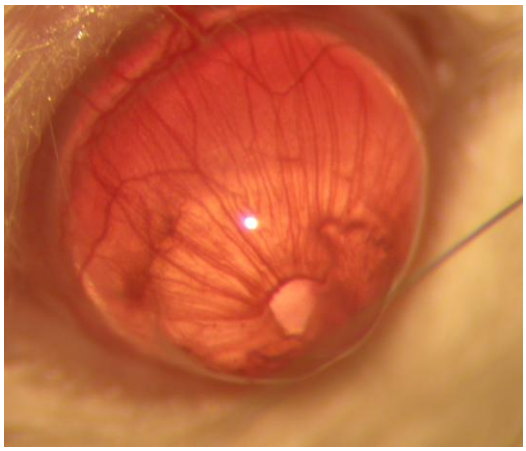


Figure 5.1 A mouse corneal allograft

Most of the graft is clear with easily visible iris detail (Grade 0). However part of the graft is opacified with loss of iris detail.

5.2. RESEARCH QUESTIONS

The aims of the work described in this chapter were to:

- evaluate the feasibility and reproducibility a pachymetry technique to measure mouse corneal thickness
- correlate increases in corneal transplant thickness with clinical and histological signs of rejection.

5.3. EXPERIMENTAL METHODS AND STUDY DESIGN

The technique of corneal transplantation, as described in detail in Chapter 2, was used in this experiment. C57BL/6 corneas were used as donors. A/J mice were used as recipients. Using the Corneogage pachymeter (Sonogage, Cleveland, USA) measurements of the donor cornea thicknes were taken pre-operatively, post-operatively and on alternate days thereafter as described in section 2.2.5 of chapter 2.

The eyes containing the first 4 grafts to reach clinical grade 4 were enucleated and embedded in OCT compound as described in section 2.4.1 of chapter 2. Following this the first 4 grafts to reach clinical grade 3 were removed and so on.

Corneal sections were cut, fixed and stained with haematoxylin. Digital images of corneal sections were captured using an Olympus digital light microscopy system. Cells in the central corneal graft were counted. Because rejected corneal allografts demonstrate variable thickness due to oedema, it was not appropriate to count the number of cells per unit area. Instead, the number of positive cells throughout the full thickness of a x100 field of the central stroma of each section was counted. Cells were counted in 3 sections per rejected graft.

4 grafts were examined in each group. The mean number of cells in the grafts was calculated.

Using a measurement tool on the Olympus software the central thickness of the frozen sections through each corneal graft was measured. Central corneal thickness was measured in 3 sections per rejected graft. 4 grafts were examined in each group. The mean the mean central corneal thicknesses were calculated.

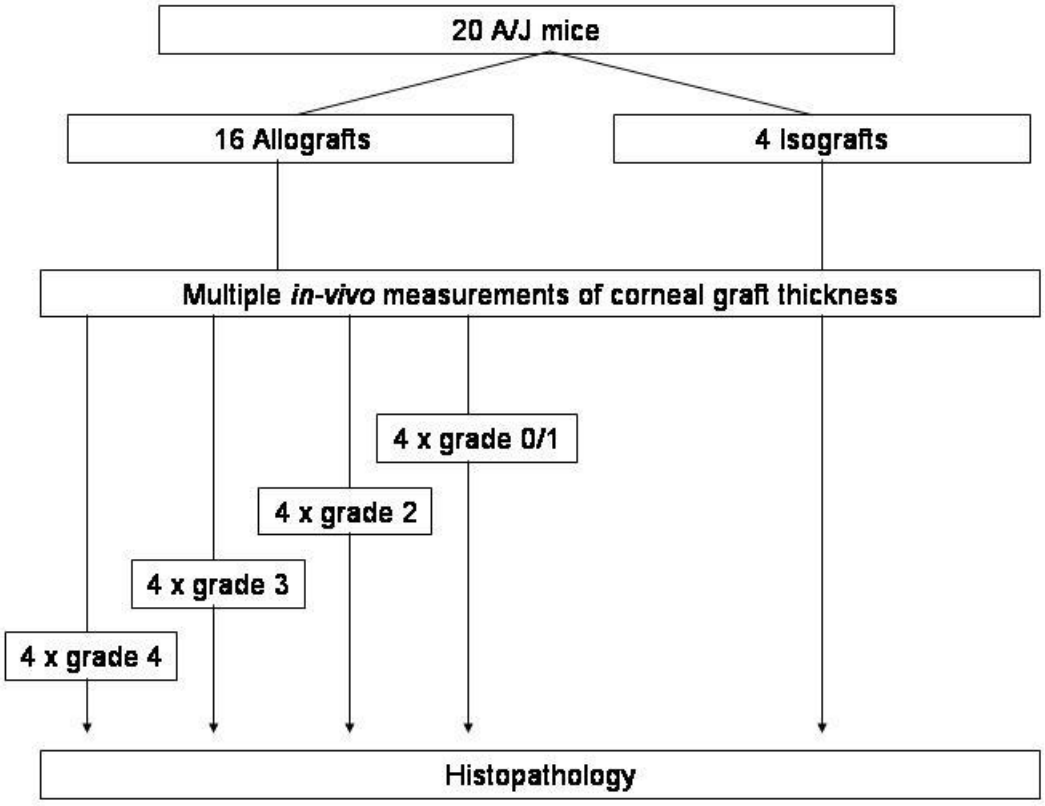


Figure 5.2 Study design

5.4. RESULTS

5.4.1. Changes in corneal thickness post-transplantation

Both allografts and isografts demonstrated an early increase in corneal thickness which peaked at post-operative day 2 to day 4 (Figure 5.4). Thereafter corneal thickness in isografts declined slowly to a level approaching that of the normal cornea whereas allografts demonstrated a subsequent rapid gross thickening. This thickening was demonstrable both *in-vivo* with pachymetry and *ex-vivo* on frozen sections (Figure 5.5).

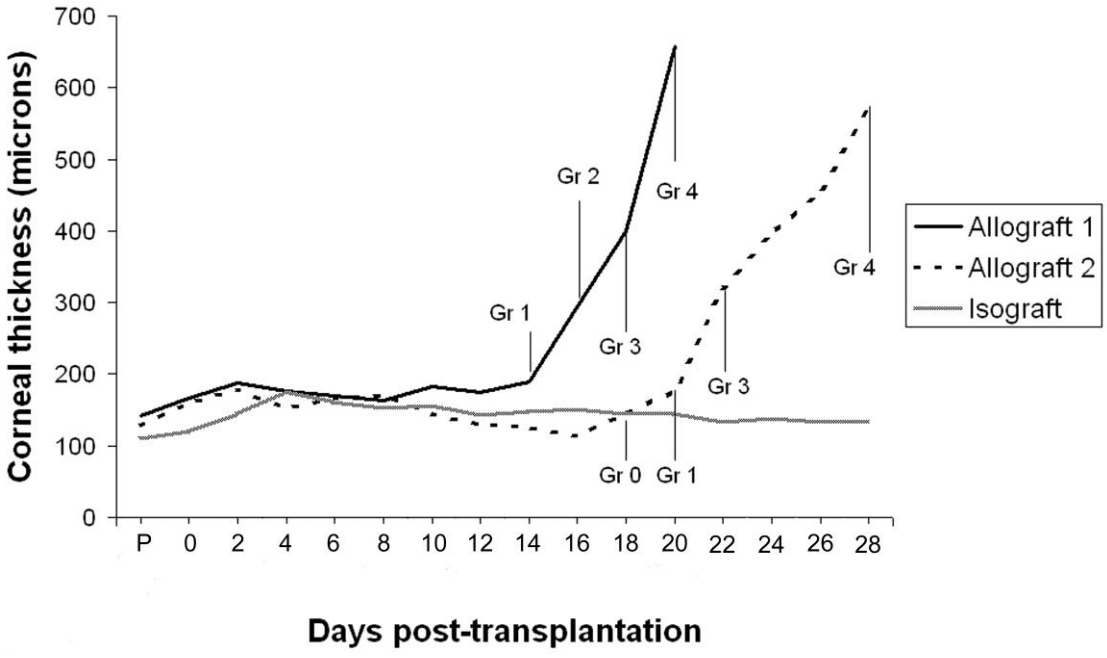


Figure 5.3 Post-operative changes in corneal graft thickness

Changes in corneal thickness in 2 representative allografts and 1 representative isograft in the first month after transplantation as measured by pachymetry.

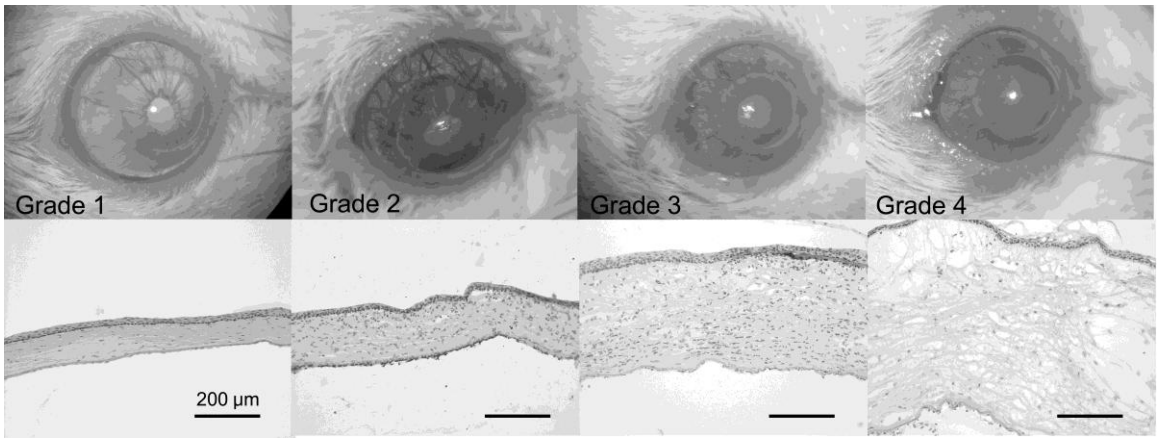


Figure 5.4 Relationship between graft clarity and corneal thickness

Graft clarity and histological features at clinical grades 1-4 following experimental corneal allotransplantation in the mouse model. Note the increases in graft thickness on histological sections of grafts at each successive grade.

5.4.2. Correlation between corneal thicknesses measured in-vivo with pachymetry and ex-vivo on frozen sections.

Comparing corneal thickness measured *in vivo* with pachymetry and *ex vivo* on frozen sections the mean bias was -45.35µm. This means that, assuming there were no artefactual changes in corneal thickness of the frozen sections, the pachymeter overestimated thickness, on average by 45.35µm. This appears to have been largely due to inaccuracies at higher corneal thicknesses. On the Bland-Altman plots measurements above 400µm appear to be more grossly overestimated by the pachymeter (Figure 5.6 A). When measurements above 400µm were excluded the mean bias was -16.44µm with 95% agreement between -60.66µm and 27.78µm (Figure 5.6 B). Visual inspection of the Bland-Altman plot can identify systematic errors in relationship between difference in measurement values and their average. i.e. one method of measurement may systematically give a higher or lower value than another (even though the two methods of measurement may correlate very well). There was no obvious systematic error up to thickness of 400 µm.

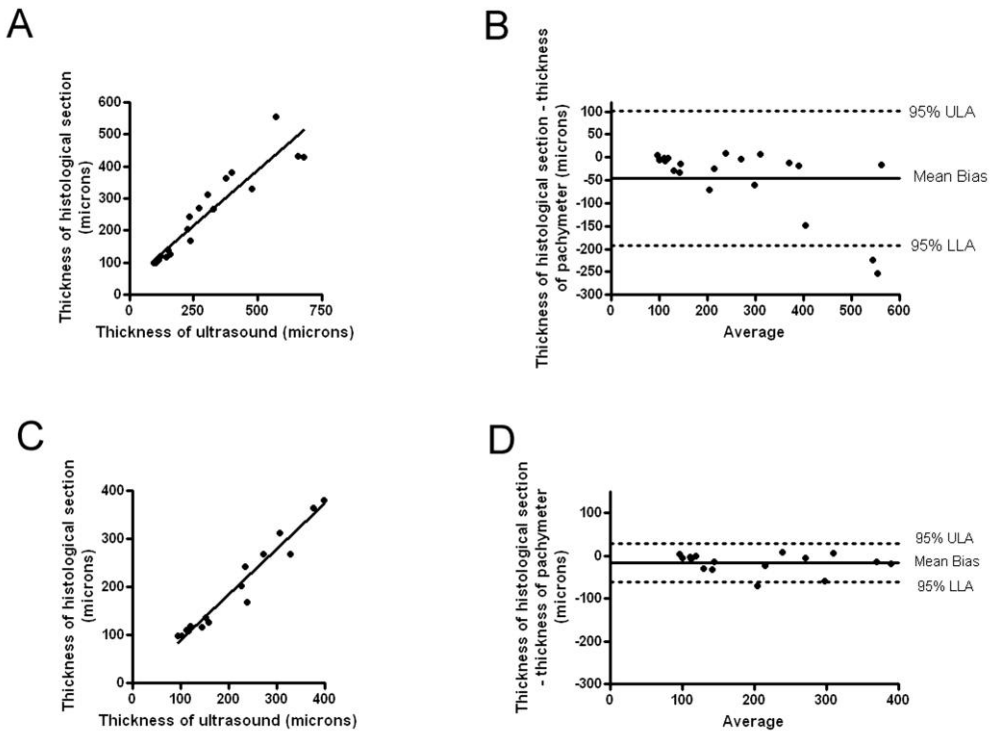


Figure 5.5 Correlation and agreement between *in vivo* and *ex vivo* measurements of corneal thickness

Correlation and agreement between corneal thickness measured in-vivo with pachymetry and ex-vivo on frozen sections as demonstrated by an X-Y scatter plot (A) and a Bland-Altman plot (B). Note the increased error for measurements of thickness greater than 400µm. When these measurements were excluded the mean bias was much reduced and no other systematic error was observed (C, D) Explain diagrams

5.4.3. Reproducibility of in-vivo measurements of corneal thickness

There was a good correlation between repeated values of corneal thickness measured with pachymetry by the same observer. (R^2=0.96 ; Figure 5.7). No systematic error was seen on the Bland-Altman plot. Mean difference was

3.96µm with 95% limits of agreement between 32.58 and -24.66µm.

There was also a good correlation between measurements by independent observers. (R= 0.92; Figure 5.8) Again no systematic error was seen on Bland-Altman plot. Mean difference was -11.35µm with 95% limit of agreement between 33.04 and -55.74µm.

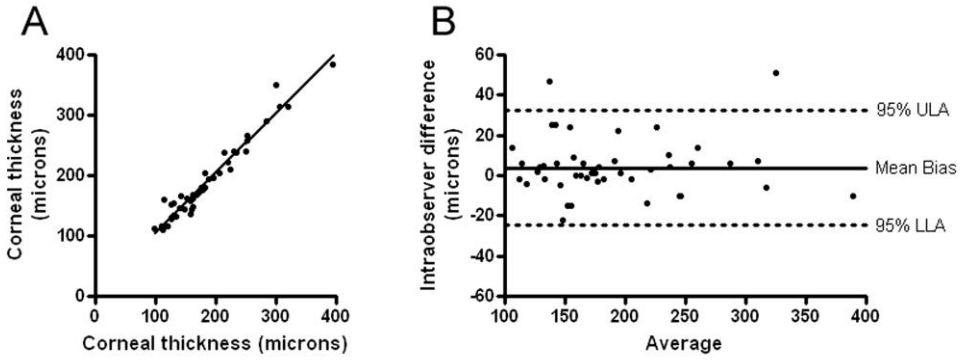


Figure 5.6 Intraobserver variation of *in vivo* measurements of corneal thickness

There was a high correlation between repeated values of corneal thickness measured with pachymetry by the same observer. (R^2=0.96 ; A). No systematic error was seen on the Bland-Altman plot(B). Mean difference was 3.96µm.

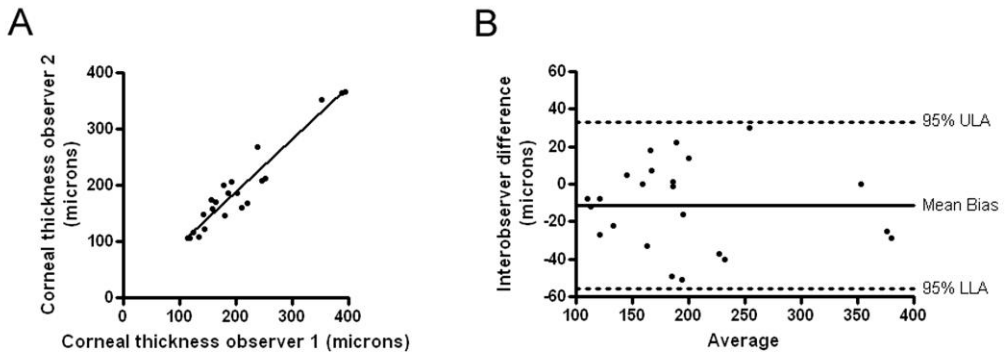


Figure 5.7 Interobserver variation of *in vivo* measurements of corneal thickness

There was a high correlation between measurements by independent observers. (R= 0.92; B) No systematic error was seen on Bland-Altman plot (B). Mean difference was -11.35µm.

5.4.4. Corneal thickness and clinical grade

The mean corneal thickness measurement by pachymetry increased with each increase in the clinical grade (Table 5.1). All grafts of clinical grades 0, 1and 2 had thicknesses of < 300µm while all grafts of clinical grades 3 and 4 had thicknesses >300µm (Figure 5.9).

Clinical grade	n of measurements	Mean corneal thickness
0	22	134.86 +/- 4.6
1	32	184.38 +/- 4.76
2	11	233.36 +/- 10.85
3	7	357.83 +/- 22.45
4	4	595.75 +/- 45.35

Table 5.1 Corneal graft thickness

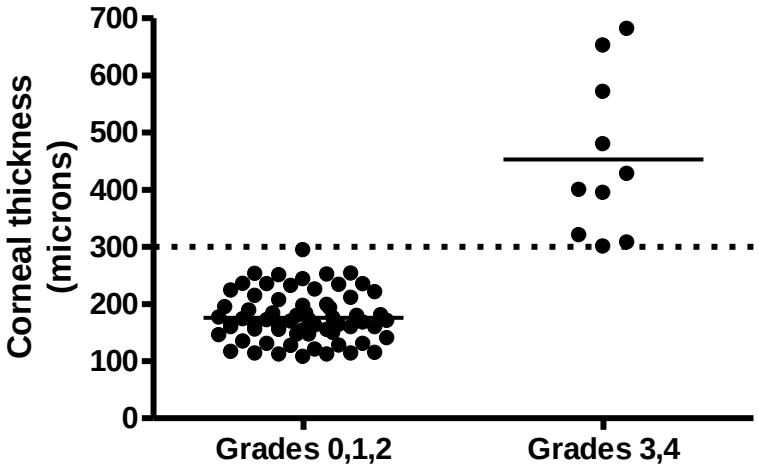


Figure 5.8 Measurements of thickness in clinically rejected and non-rejected corneal grafts.

Median graft thickness was significantly greater in rejected (grades 3 and 4) versus non-rejected (grades 0,1 and 2). P<0.0001. All clinically non-rejected grafts had thicknesses of <300µm. All clinically rejected grafts had thicknesses ≥300µm. Horizontal bars represent medians.

5.4.5. Correlation of graft thickness with number of stromal cells

The increase in corneal thickness seen as graft opacification progresses from grade 0 to grade 2 is associated with increased cellularity of the graft stroma (Figures 5.5, 5.10). Further increases in thickness beyond grade 2 / 300µm did not appear to be associated with further increases in graft stromal cellularity (Figure 5.10).

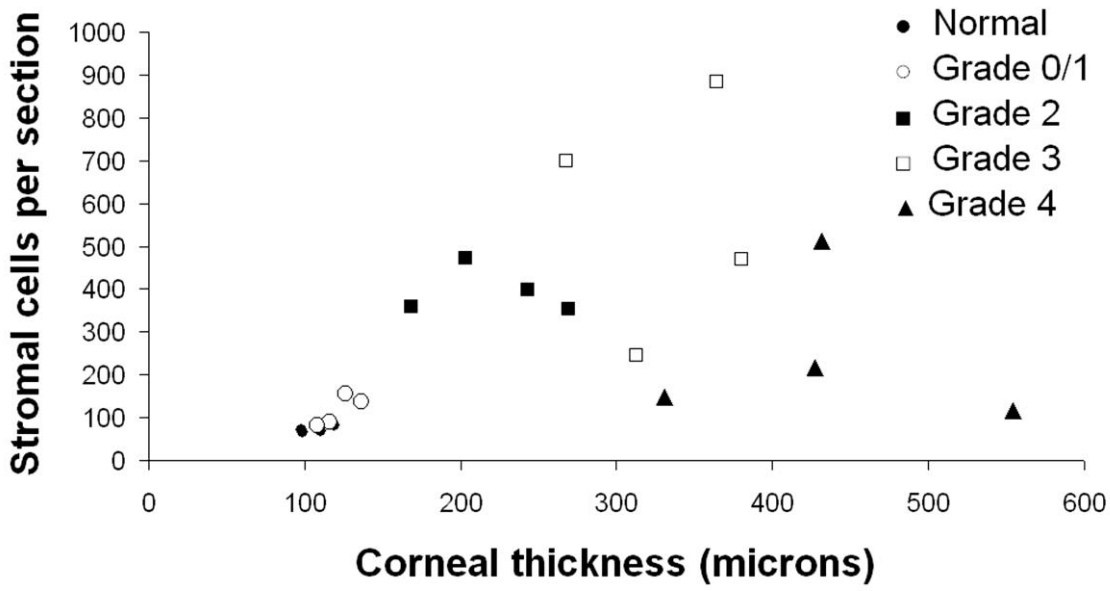


Figure 5.9 Correlation of graft thickness with number of stromal cells

The increase in corneal thickness seen as grafts progress from grade 0 to grade 2 is associated with increased cellularity of the graft stroma. Further increases in thickness beyond grade 2 / 300µm do not appear to be associated with further increases in graft cellularity.

5.5. DISCUSSION

A key shortcoming of the mouse model of corneal transplantation is imprecision in diagnosis of rejection. In rabbits and rats, endothelial precipitates can in most graft recipients be directly visualised as a clear rejection end-point(Katami, 1991), especially valuable in studies of interventions to delay or accelerate rejection; this diagnostic feature is rarely seen in the smaller mouse eye. Previously reported criteria for diagnosis of mouse graft rejection rely on subjective assessment of graft clarity.

The experiment described in this chapter has shown that mouse central corneal graft thickness may be reliably measured *in vivo* up to thickness of 400 µm using a clinical pachymeter, and that graft thickness increases during allograft rejection. In this study a small number of measurements in grafts of grade 4 opacity suggest that pachymeter measurements of greater than 400 µm are less reliable. This is not of undue concern, as these measurements were made in grafts examined later than the clinically observed onset of rejection, a situation that usually does not occur in experimental studies of graft rejection. Early transient post-operative graft thickening was seen in both allografts and isografts. This thickening, which has also been described in the rabbit model of corneal transplantation(Rayner et al., 2000), occurs too early to be mediated by a specific immune response and is most likely due to surgery-induced, alloantigen-independent inflammation. We found rejection-associated graft thickening to be a rapid process, with grafts moving from baseline to maximal thickness in about one week.

The finding that all grafts of grades 0, 1 and 2 had thicknesses of <300 µm whereas all grafts of grades 3 and 4 had thicknesses >300 µm suggests that using an increase in graft thickness to 300 µm to identify established rejection would be as good as the existing method of grading by an experienced observer. Use of pachymetric graft measurements would have a number of advantages over other criteria to diagnose rejection. First, this technique would provide an

objective measure of graft thickness, eliminating some observer bias. In this context it is notable that very few published studies of rodent corneal graft rejection report masking of the examining investigator with respect to experimental intervention groups. Second, it would standardise the diagnosis of experimental graft rejection, allowing better comparison of survival results between different laboratories. Third, it can be used by inexperienced observers. The mean interobserver bias of 11.35 μm is insignificant in the context of increases in graft thickness from 134 μm (grade 0) to 300 μm (at rejection).

In published studies and earlier work from our own laboratory, graft opacity grade 3 has been used as an end-point because the loss of iris detail at this grade is a more definite feature than the much more subjective signs designating opacity grades 1 and 2. Notwithstanding, it is clear that grafts at grade 2 contain infiltrating immune cells and are probably undergoing rejection (Figures 5.4, 5.10).

It is of interest that there was no linear correlation between the increase in graft thickness and the number of graft-infiltrating cells during rejection. Early thickening of the graft was associated with a large graft infiltrate. Inflammatory cells were seen adhering to the endothelium on histological sections of grafts with early (grade 2) thickening (Figure 5.5). Thereafter the graft continued to thicken without further increases in graft-infiltrating cells. Grafts at opacity grade 4 contained surprisingly few cells. Given the rapid nature of graft thickening, this suggests that cellular immune-mediated inflammation in the graft stroma during rejection in this model is quite short-lived, and that endothelial injury and decompensation is a more significant determinant of corneal thickness change in the late phase of rejection. It is easier to study the cellular mediators of stromal cell rejection than those of endothelial rejection and human and animal studies have largely relied on the analysis of stroma-infiltrating cells in sections of rejected corneal grafts. It has been assumed, not unreasonably, that those cells mediating stromal rejection are the same as those mediating endothelial rejection. Rejected human grafts are invariably studied at quite a long interval after onset of rejection. In mice the numbers of stroma-infiltrating cells appear to

peak early after onset of rejection and decrease in established rejection. If this were to occur in human rejection then further questions would be raised as to the relevance of the inflammatory cells seen on histopathological examination of graft stroma to the process of endothelial rejection.

In experimental corneal pachymetry may allow discrimination between (i) onset of rejection and (ii) maximal graft inflammation, either of which may be of particular interest to investigators as an experimental endpoint. We found that graft inflammation was maximal when thickness was close to 300 µm rather than much higher. Use of a graft thickness cut-off of 300 µm for rejection would therefore coincide with maximal graft inflammation, a measurement of value in studies of effector mechanisms of graft rejection. On the other hand a graft thickness of greater than 200 µm could be used to diagnose the earliest onset of rejection. This lower figure is based on our finding of large numbers of graft-infiltrating cells in specimens at thickness between 200 µm and 300 µm. A potential disadvantage of using graft thickness of 200 µm would be the risk of false positives, given that i) some grafts show early post-surgery (presumably alloantigen-independent) thickening to approximately this level and ii) the fact that there is some inter/ intra observer error. Whichever cut-off is used as an experimental endpoint, I feel that graft pachymetry will be a useful tool in experimental corneal transplantation in small animals.

6. CHAPTER 6: Aqueous humour alloreactive cell phenotypes, cytokines and chemokines in human corneal allograft endothelial rejection

6.1. INTRODUCTION

6.1.1. Biopsy of transplanted organs

Allogeneic rejection of transplanted tissues is a complex immunological process culminating in the infiltration and destruction of the tissue by host leukocytes. The need for tissue biopsy to diagnose rejection in vascularised organ grafts has allowed study of the phenotype of graft-infiltrating cells(Hancock et al., 1983) during acute rejection of these grafts. As long ago as 1958, histological analysis of rejecting skin allografts identified macrophages and lymphocytes as the predominant mediators of immune rejection in that tissue(Brent et al., 1958). Biopsy of corneal tissue is not performed or necessary during acute corneal graft rejection because graft transparency would be compromised by scarring at the surgery site and corneal graft rejection can be diagnosed by direct observation of the eye. Accordingly, surprisingly little information is available on the cellular and molecular mediators of acute corneal graft rejection in humans. Almost all information on the effector components in the early phase of acute corneal graft rejection comes from animal models

6.1.2. Phenotype of graft-infiltrating cells during rejection

In the experiments described in chapter 3 I identified $CD4^+$ T lymphocytes, $CD8^+$ lymphocytes, and macrophages in acutely rejected mouse corneal allografts. These data are consistent with reports describing corneal graft rejection in other rabbits and rats(Williams et al., 1992,Larkin et al., 1997b). Published histopathological studies of replaced human grafts that have failed following rejection also report $CD4^+$ T lymphocytes, $CD8^+$ lymphocytes, and macrophages in the graft but these studies describe cellular changes at a long interval after observed rejection onset and prolonged topical steroid administration(Larkin et al., 1997a). Data from my experiments desribed in Chapter 5 demonstrated variation in the numbers of graft-infiltraing cells in relation to the clinical course of

rejection with considerable reduction in the number of graft-infiltrating cells in the later stages of rejection. If such a phenomenon were to occur in humans, then the results of pathological studies of rejected corneal grafts would not reflect accurately the cellular events during acute rejection. In addition pathological studies on rejected human corneal grafts provide information on cells infiltrating the graft stroma only and no information on the alloreactive cells in the aqueous humour which interact directly with the endothelium of donor cornea, itself critical for maintenance of graft transparency.

6.1.3. Aqueous sampling and analysis of inflammatory cells in human aqueous

Cells in the aqueous are accessible to sampling and diagnostic anterior chamber sample removal has been shown to be a safe procedure in corneal transplant rejection and in uveitis(Van der and Rothova, 1997,Calder et al., 1999). Flow cytometry has been used by a number of groups to identify subtypes of leukocytes in aqueous humour during acute anterior uveitis. In 1986 Deschenes desribed the use of flow cytometry to measure proportions of cytotoxic T-, helper T- and B lymphocytes in aqueous humour of patients with acute anterior uveitis (Deschenes et al., 1986). Deschenes and other early adopters of flow cytometry for aqueous analysis were predominantly interested in lymphocytes subsets(Wang et al., 1995). Later Muhaya et al and Calder et al evaluated proportions of other leukocytes as well as lymphocyte subsets in anterior uveitis and consistently found monocytes(Calder et al., 1999,Muhaya et al., 1998). Curnow looked at lymphocytes in more detail measuring expression of the chemokine receptor CXCR4 on lymphocytes in aqueous during acute anterior uveitis and found that this receptor was up-regulated in patients treated with glucocorticoids(Curnow et al., 2004b).

Reinhard and colleagues have analysed cells in aqueous humour during acute corneal graft rejection using cytospin microscopy(Reinhard et al., 2002). This

paper demonstrated that aqueous from patients who are undergoing endothelial rejection contains macrophage / monocytes, lymphocytes and granulocytes(Reinhard et al., 2002) but it did not describe the relative quantity of each subset.

Using the flow cytometry machine in our laboratory (Partec) expression of 4 different proteins on the surface or inside (intracellular staining) cells could be measured simultaneously. Given the evidence for a role for $CD4^+$ cells in corneal graft rejection it was tempting to assume that these cells would be found in aqueous humour, to use one antibody against CD4 and to use the other 3 available markers/ colours to measure functional determinants of $CD4^+$ activity e.g. costimulatory molecule ligands (CD28, CD40) or markers for the regulatory T cell subset (CD25, FoxP3). In the end I decided that, because there were so few data available on aqueous cell phenotypes during corneal graft rejection, it would be best to use antibodies against CD4, CD8 and CD14 to try to answer the most fundamental questions. (see chapter aims). I considered using the fourth colour to try to identify eosinophils (I had hoped to recruit some patients with graft rejection and a history of atopy or allergic eye disease). This would have allowed a direct comparison with the results of cell phenotypes in rejected mouse corneal grafts. However, when I learned that no single cell surface marker could be used to identify human eosinophils I disregarded this plan. For the fourth colour I also considered using an antibody against natural killer cells which have been found in aqueous of rats during corneal graft rejection. In order to quantify proportions of leukocytes staining with each marker I needed to be able to identify which events were leukocytes. In analysis of peripheral blood leukocytes can be identified in characteristic clusters of events on the FSC/SSC plot. I was not sure whether this would be the case in aqueous so I decided to use my final colour to stain for the pan-leukocyte marker, CD45, which would confirm the presence (or absence) of leukocytes in the aqueous samples.

6.1.4. Analysis of inflammatory cytokines and chemokines in human aqueous

The expression of pro-inflammatory cytokines and chemokines in corneal tissue during corneal allograft rejection has been studied in experimental animals using RT-PCR and ribonuclease protection assay (Torres and Kijlstra, 2001,Pillai et al., 2008a,Yamagami et al., 1999,King et al., 2000). There are no reports of analysis of aqueous cytokines and chemokines in animal models of corneal transplantation. This is probably due to the fact that, in the species of animals usually used as recipients (rodents), the volume of aqueous humour is small. The chemokines found with greatest consistency in the analyses of corneal/ ocular tissue were MCP-1, RANTES, MIP-1α and IP-10(Yamagami et al., 1999). I therefore decided to measure these chemokines in human aqueous. In addition I decided to measure Eotaxin. (At the beginning of the study I had hoped to recruit some graft rejection patients with a history of atopy/ allergic eye disease and whilst I did not have a suitable cellular marker for eosinophils for use in the cellular analysis, I felt that expression of Eotaxin which is chemotactic for eosinophils would be relevant and worth measuring).

Cytometric bead array has been used for analysis of aqueous humour cytokines in patients with uveitis(Curnow et al., 2005) and appears to be a reliable method for the estimation of protein concentration in aqueous humour, agreeing reasonably well with ELISA measurements(Ooi et al., 2006). One previous study of human aqueous during corneal allograft rejection was published while my own study was ongoing. In this study Funding and co-workers used multiplex beads to quantify some of the inflammatory markers in aqueous during acute human corneal graft rejection(Funding et al., 2006). The chemokines MCP-1,IL-8, MIP-1β were found in human aqueous during corneal graft rejection and not in normal aqueous. In addition they found expression of the following cytokines: IL-1β, IL-2, IL-4, IL-6, IL-7, IL-10, IL-12p70, IL-13, IL-17, IFN-γ and TNF-α.

6.1.5. Chapter aims

The aims of the work presented in this chapter were to:

- Establish whether leukocytes could be identified in human aqueous samples during acute rejection of corneal graft endothelium.
- Identify and quantify the proportion of leukocytes in aqueous humour during rejection that stained positive for CD4, CD8 and CD14.
- Compare the proportions of $CD4^+$, $CD8^+$ and $CD14^+$ leukocytes in aqueous humour with those in peripheral blood.
- Measure the concentrations of cytokines and chemokines in aqueous humour in normal patients and patients with acute corneal graft endothelial rejection.
- Measure the concentrations of cytokines and chemokines in serum in normal patients and patients with acute corneal graft endothelial rejection.

6.2. EXPERIMENTAL METHODS AND DESIGN

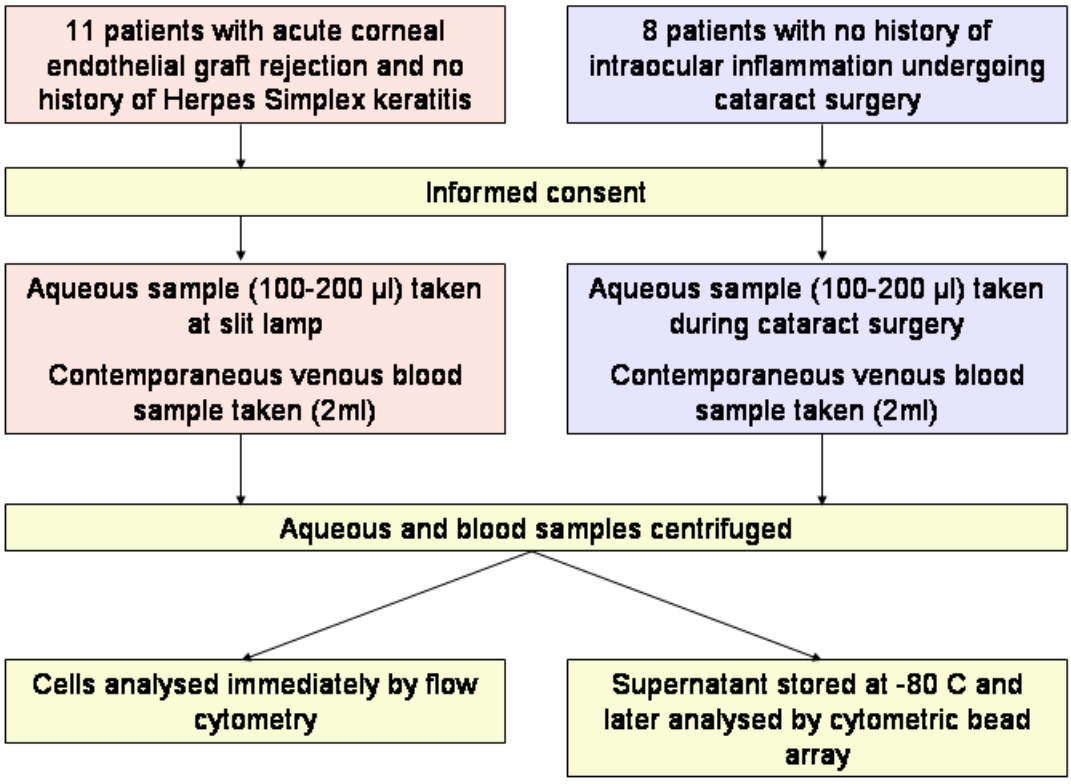


Figure 6.1 Study design

6.2.1. Aqueous humour sample collection

Aqueous humour (100 -200µl) samples were obtained via anterior chamber paracentesis with either an insulin syringe or an aqueous micropipette (Figure 6.2; Becton Dickinson, Oxford, UK). Topical proxymetacaine was instilled in the patient's eye. A drop of povidone iodine 5% was instilled 5 minutes prior to sampling. For patients with corneal graft rejection an eyelid speculum was inserted to fix the eyelids in position during aqueous sampling. The patients

head was positioned on the chin rest of the slit lamp and the needle was inserted through the temporal cornea into the anterior chamber taking care to avoid intersecting with the needle tip any corneal vessels near the entry site. Aqueous was slowly withdrawn whilst observing the consequent shallowing of the anterior chamber. The needle was withdrawn from the anterior chamber at a time deemed safe to avoid any contact of the needle tip and iris/ lens. This typically led to a yield of between 100-200µl of aqueous humour. The aqueous was transferred to an EDTA-coated Eppendorf tube and taken directly to the laboratory for immediate analysis. Simultaneous peripheral venous blood samples (2 ml) were taken from each patient into an EDTA- coated tube. Control aqueous samples were taken after the initial corneal incision during cataract surgery.

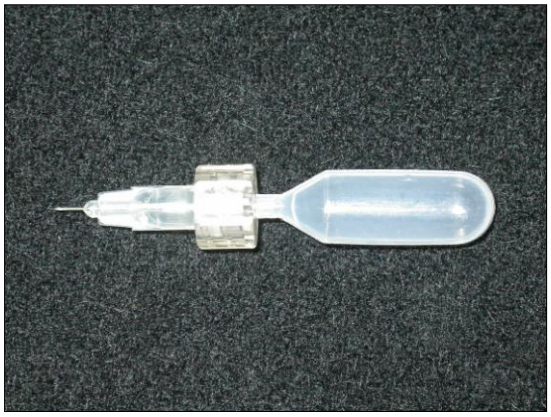


Figure .6.2 Aqueous micropipette

6.2.2. Flow Cytometry- Acquisition

Acquisition of flow cytometry data for peripheral blood has been described in detail in chapter 2. Aqueous samples were handled as follows:

Aqueous humour (AH) samples were transferred to EDTA-coated microtubes and centrifuged at 300g for 5 minutes at 4°C. Thereafter the cell-free supernatant

was collected and stored in aliquots at -70°C for later analysis by cytometric bead array. The cell pellet was resuspended in 200µl PBS and divided evenly into two tubes. One tube was stained with 5µl each of fluorochrome-labelled anti-CD45, anti-CD4, anti-CD8 and anti-CD14 monoclonal antibodies (antibodies described in detail in Chapter 2). The other was stained with fluorochrome-labelled isotype-matched control antibodies (all antibodies have been desrcribed in detail in Chapter 2). Cells were incubated with antibodies for 40 minutes in darkness at room temperature.

Then cells were centrifuged at 300g for 5 minutes at 4°C and resuspended in PBS for 5 minutes. They were then centrifuged at 300g for 5 minutes at 4°C and finally resuspended in PBS before being analysed immediately by 4-colour flow cytometry without fixation.

6.2.3. Flow Cytometry- Analysis

For peripheral blood samples a gate was drawn manually around the leukocyte cluster as defined by their physical properties on the FSC/SSC scatter plot and the resulting events were analysed by creating quadrants to determine the percentages of CD45+ events which were also positive for CD4, CD8 and CD14 respectively. Quadrant analysis allows the determination of proportions of double-stained cells in a scatter plot of one colour versus another (Figure 6.3) In samples where a leukocyte cluster could be identified, aqueous humour was analysed in the same way.

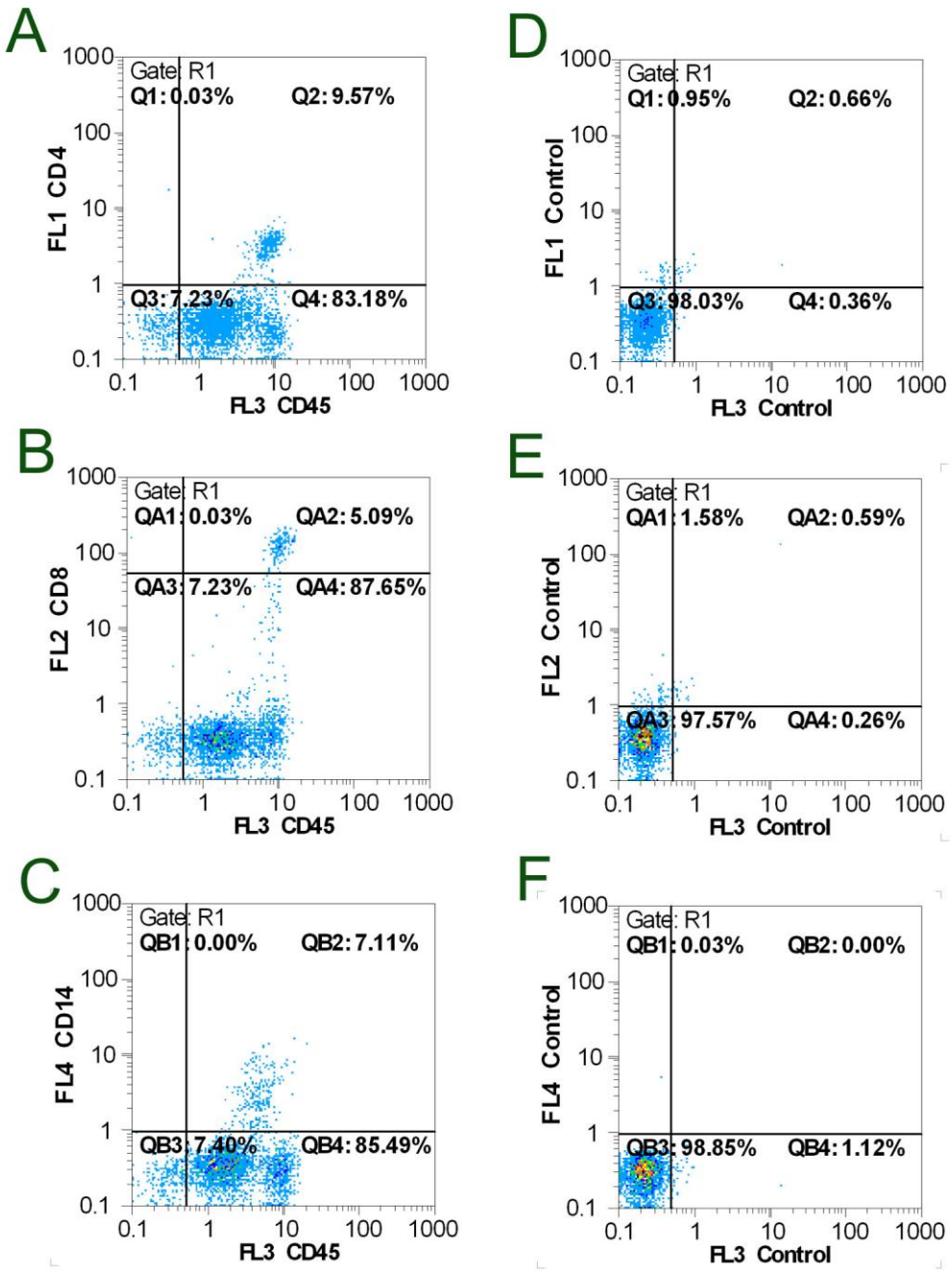


Figure 6.3. Quadrant analysis of double-stained cells

The proportion of $CD45^+CD4^+$ (A), $CD45^+CD8^+$ (B) and $CD45^+CD14^+$ (C) double positive cells could be measured using quadrant analysis.
Plots for peripheral blood stained with isotype controls are shown in panels D, E and F. The cells show low fluorescence with few positive events.

6.3. RESULTS

6.3.1. Patient demographics

Patients' diagnoses and demographics are summarised in Table 5.1.

	Sex	Age	Primary corneal diagnosis
1	F	62	Pseudophakic bullous keratopathy (PBK)
2	F	85	PBK
3	M	76	Fuchs endothelial disease (FED)
4	F	73	PBK
5	M	65	PBK
6	F	81	FED
7	M	83	PBK
8	M	51	PBK
9	F	72	FED
10	M	65	Microbial keratitis
11	M	38	Keratoconus

Table 6.1 Age, sex and primary diagnoses of patients with corneal graft rejection

6.3.2. Sample collection and rejection outcome

There were no complications of aqueous humour paracentesis. Following paracentesis patients were treated with hourly topical dexamethasone 0.1% and chloramphenicol drops four times per day to the involved eye. As determined by elimination of intraocular inflammation, rejection was reversed in all 11 cases. In two patients the grafts did not recover transparency even though anterior chamber inflammation was eliminated, presumably due to extensive endothelial cell loss by the time of reversal, and these grafts were deemed to have failed.

6.3.3. Identification of leukocytes in aqueous of patients with rejection

The volume of aqueous humour collected from each patient was approximately 100 to 200µl. In all samples many events were visible on the FSC/SSC scatterplot. In 8 of the 11 aqueous humour samples there was an observable cluster of events on these plots (Figure 6.4;A). Aqueous stained with PerCP-labelled anti-CD45 antibody revealed a second peak in fluorescence suggesting that some of the events on the scatter plot were cells expressing CD45 i.e. were leukocytes. By gating on the $CD45^+$ events it became clear that the $CD45^+$ events corresponded with the visible cluster of events on the scatterplot (Figure 5.3). The median number of $CD45^+$ events observed per sample was 199 (range 81-837). Apart from the $CD45^+$ cluster there tended to be a lot of other events on the scatterplot which were $CD45^-$ (Figure 6.4). In 3 samples no observable $CD45^+$ cluster of events was visible on the FSC/SSC scatter plot although in each of these cases cells had been observed in the anterior chamber at the time of diagnosis of rejection. I can only conclude that the cells in these samples were lost during the staining process.

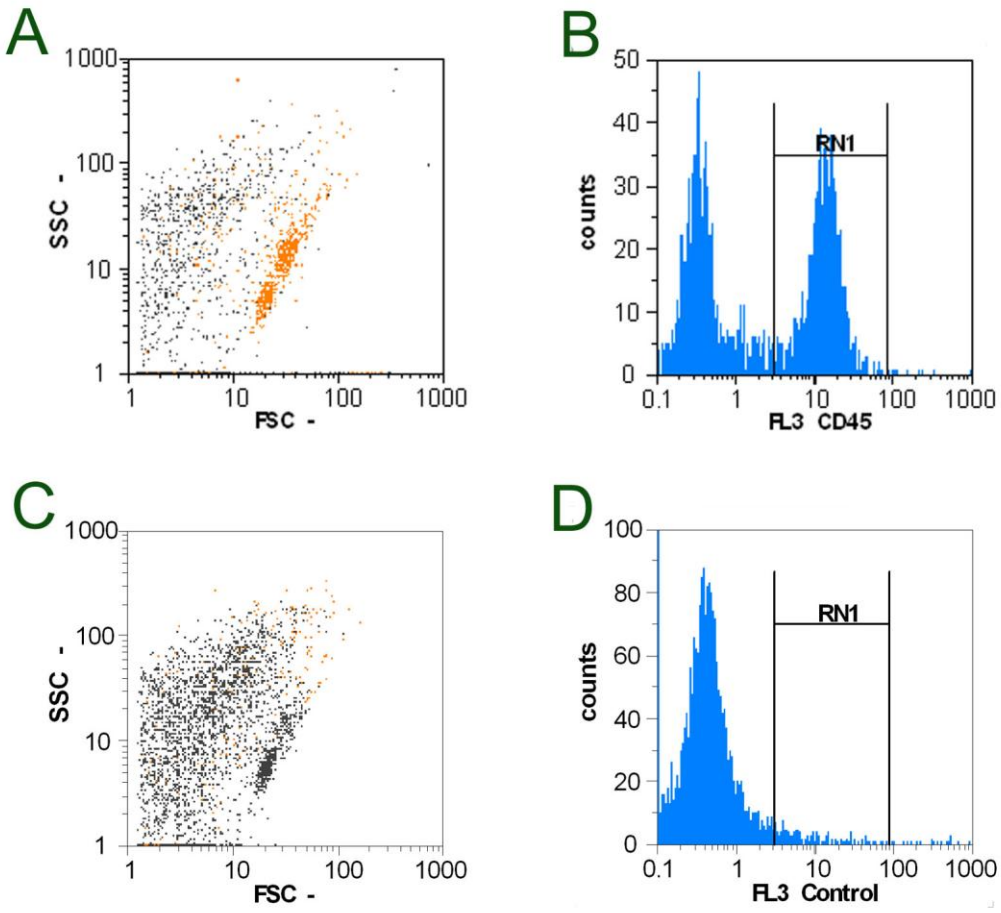


Figure 6.4. Identifying leukocytes in human aqueous

A cluster of events is visible on the scatterplot (A). Gating on the $CD45^+$ events (Region gate RN1 in panel B) confirms that the events within the cluster on the scatterplot (seen in red in A) are $CD45^+$ i.e. are leukocytes.
Exposure to PerCP-labelled IgG1 (isotype control for anti-CD45 antibody) does not lead to a shift in fluorescence or a second peak in fluorescence as seen in Panel B. This shows that the second peak in fluorescence in aqueous stained with anti-CD45 antibody is due to specific affinity for the antibody for these cells.

6.3.4. Analysis of normal aqueous

As in rejection samples many events were seen on the scatterplot which were predominantly CD45$^-$. As in control staining of aqueous during rejection, a small and similar amount of positivity was seen for both CD45 and isotype control. The typical cluster of events on scatterplot which represented cells in rejection samples were not seen (Figure 6.5).

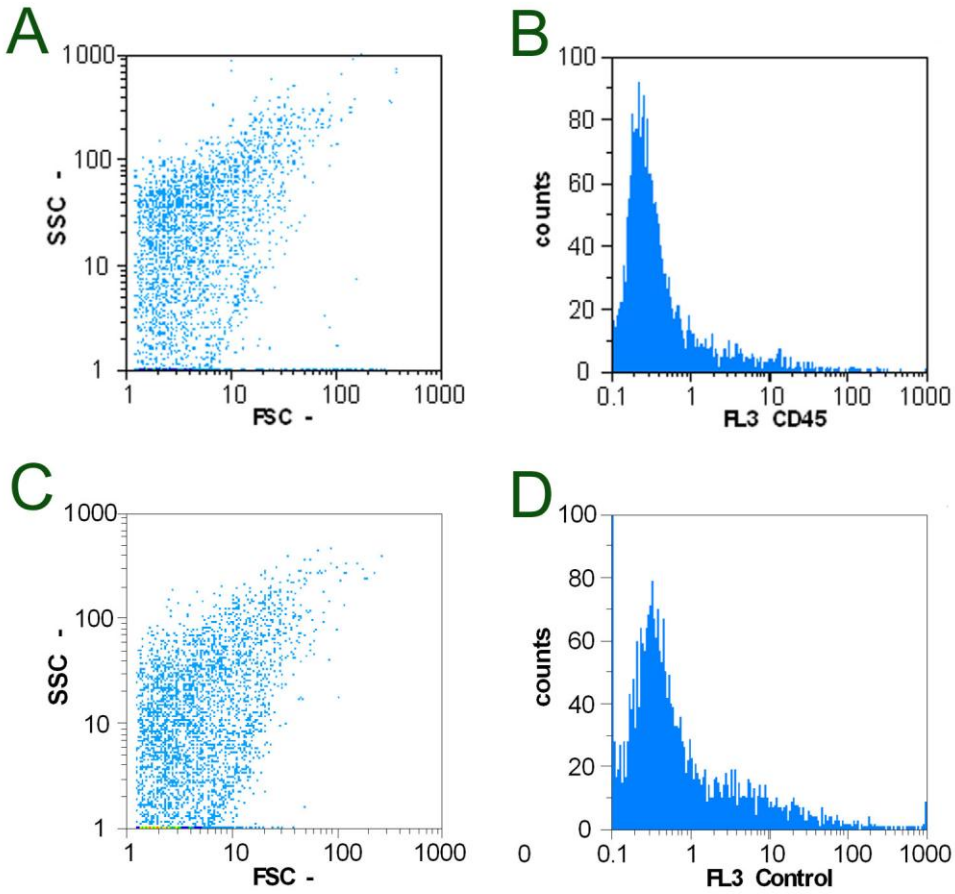


Figure 6.5. Flow cytometry of normal aqueous

Many events are seen on the scatter plot (A). These events are CD45$^-$. There is no difference in staining between anti-CD45 (B) and its isotype control (C,D).

In some control samples a cluster of events was visible on the scatterplot. Gating on these events demonstrated that they were $CD45^-$ i.e. were not leukocytes (Figure 6.6.) One possibility is that they represent cellular debris released from the iris in response to the initial paracentisis during cataract surgery.

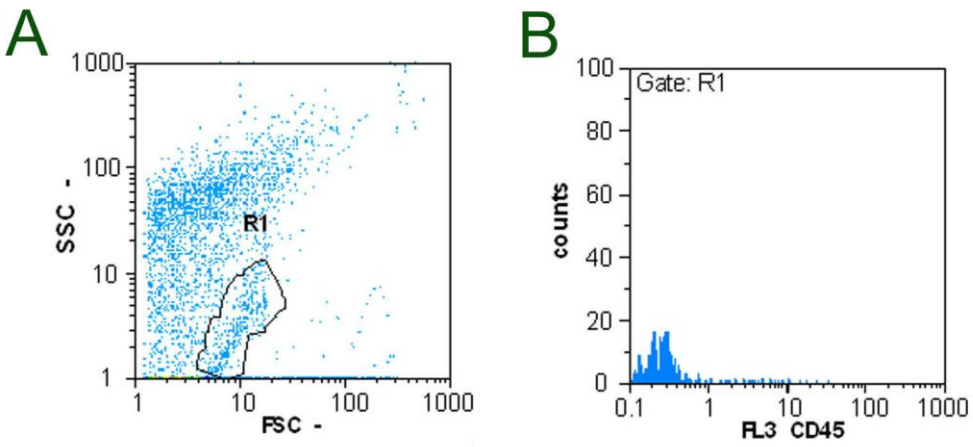


Figure 6.6. CD45 staining in normal aqueous

In some control samples of aqueous, taken during routine cataract surgery, a cluster of events was visible on the scatter plot. These are seen in region R1 in panel A. Gating on these events revealed that, unlike the events seen during graft rejection, they were $CD45^-$.

6.3.5. Aqueous and peripheral blood cell phenotypes

To quantify proportions of the leukocyte subsets a gate was drawn manually around the leukocyte event cluster on the scatter plot. The events within this gate were confirmed as $CD45^+$ and only these events were used in the analysis. The relative proportions of $CD45^+CD4^+$, $CD45^+CD8^+$ and $CD45^+CD14^+$ events were calculated using quadrant analysis (Figure 6.7). Isotype control staining of aqueous showed a small amount of positive staining (Figure 6.7) which was most likely due to retained antibody (samples were washed only once to minimise cell loss).

T-lymphocytes ($CD45^+CD4^+$ and $CD45^+CD8^+$) and monocytes/macrophages ($CD45^+CD14^+$) were present in the aqueous of all patients with endothelial rejection (Fig 6.7). The percentages of each phenotype of white blood cells in aqueous and peripheral blood are presented in Table 6.2. The majority (58%) of immune cells in aqueous during rejection were $CD14^+$. This percentage of monocytes / macrophages in aqueous during rejection was significantly higher than that in peripheral blood (median value of 63.0% v 9.3%; $p<0.0001$; Figure 6.8). This was also true of $CD8^+$ cells (median value of 18.5% v 9.9%; $p= 0.036$). Although the percentage of $CD4^+$ cells was higher in aqueous during rejection than in peripheral blood (median value of 24.9% v 14.5%), this difference was not statistically significant. The percentage of $CD14^+$ cells in peripheral blood during rejection was significantly higher than that in control samples (median value of 9.3% v 7.3%; $p=0.036$). There were no significant differences between the percentages of either $CD4^+$ or $CD8^+$ cells in peripheral blood in rejection and in controls respectively (Fig 6.9).

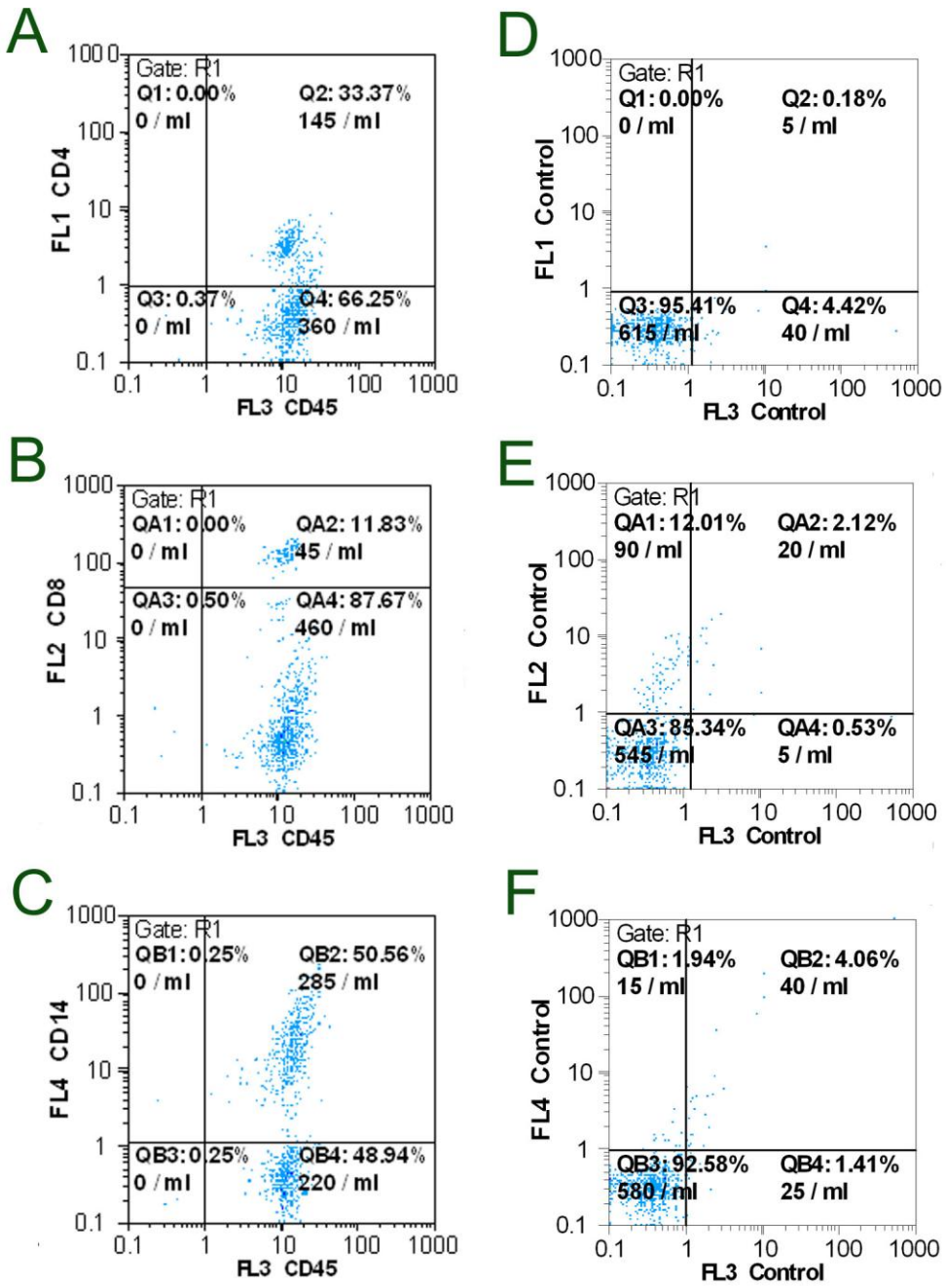


Figure 6.7 Flow cytometry of aqueous humour

These $CD45^+$ leukocytes were further analysed in terms of their expression of CD4, CD8 and CD14

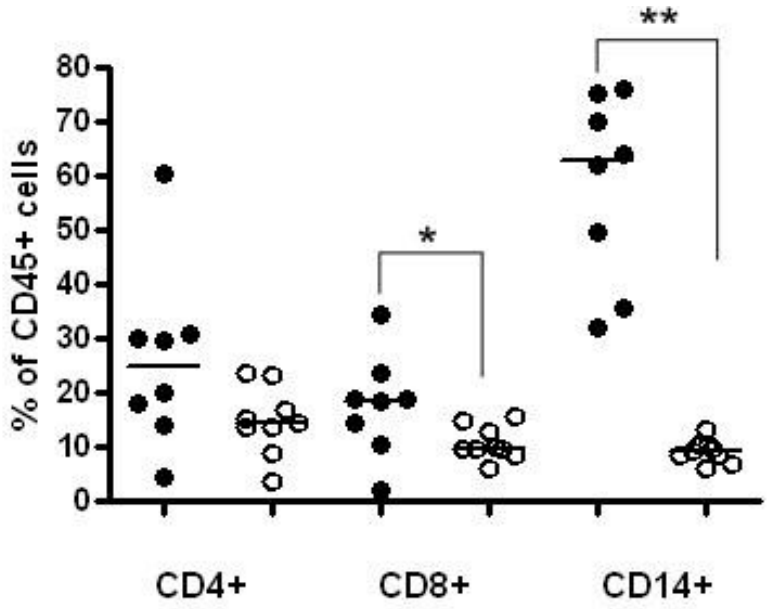


Figure 6.8 Phenotypes of inflammatory cells in aqueous during rejection

Points shown are the values for each cell phenotype in aqueous humour (n=8, cells were not seen on flow cytometric analysis of 3 of the 11 samples) and peripheral blood (n=11) in patients undergoing acute endothelial rejection. CD14+CD45+ were the most frequent cells in aqueous humour during rejection followed by CD4+CD45+ and CD8+CD45+. The respective proportions of CD14+CD45+ and CD8+CD45+ in aqueous (●) were significantly higher than those in peripheral blood (○) (*$p<0.05$, **$p<0.0001$). Horizontal lines are at the median values.

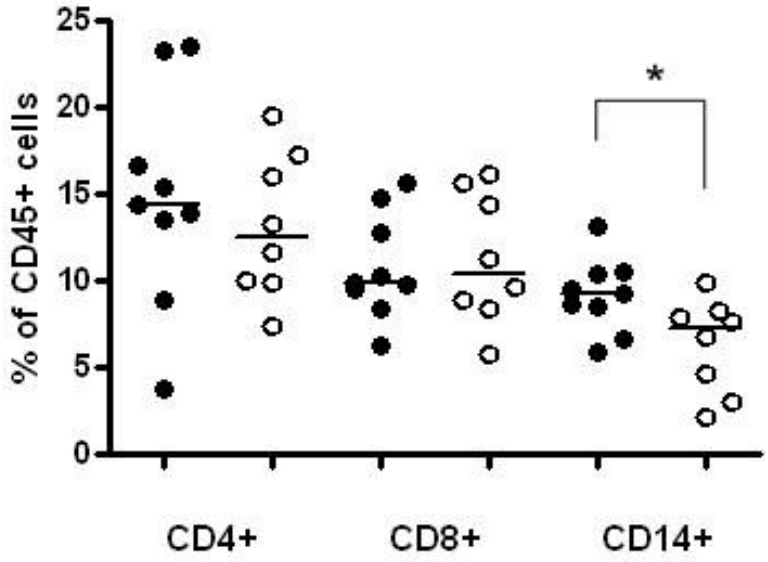


Figure 6.9 Phenotypes of inflammatory cells in peripheral blood during rejection

The proportion of CD14+CD45+ cells in peripheral blood was significantly higher during rejection (●) than in controls (○) (*$p<0.05$). Negative controls samples were aqueous samples taken at the time of elective cataract surgery. Horizontal lines are at the median values.

	$CD45^+CD4^+$	$CD45^+CD8^+$	$CD45^+CD14^+$
Aqueous rejection	24.89	18.49	63.04
Aqueous control	No cells	No cells	No cells
Blood rejection	14.45	9.86	9.33
Blood control	12.48	10.45	7.25

Table 6.2 Phenotypes of cells found in aqueous humour and peripheral blood expressed as median % of all CD45+ cells.

See also Figures 5.8 and 5.9 for individual data points

6.3.6. Cytokines and chemokines in aqueous and peripheral blood

The cytokines and chemokines tested are listed in table 5.3. Only the cytokine IL-6 and the chemokines MCP-1 and IP-10 were found at high levels in aqueous during endothelial rejection (Figure 6.10). Expression of each of these three molecules was found also in control samples, but the levels during rejection were significantly higher than those in controls. No expression of MIP-1a or eotaxin was found in the aqueous humour of control patients, but low levels of both were seen in aqueous during rejection (summarised in Table 6.3). Levels of IFN-γ and TNF-α were very low or below the levels of detection.

No significant differences were found in the expression of cytokines and chemokines in peripheral blood between rejection and control samples (summarised in Table 6.4). It is of interest that very high levels of RANTES were found in peripheral blood of both groups (median 4097.1 pg/ml in rejection and 4827.2 in controls).

	Rejection		Control		
	Median (pg/ml)	Range (pg/ml)	Median (pg/ml)	Range (pg/ml)	p value
IL-2	0	0	0	0	_
IL-4	0	0	0	0	_
IL-6	5399.29	8.58-46354.69	24.67	0-93.69	0.0015
IFN-γ	0	0-5.02	0	0	_
TNF	0	0	0	0	_
MCP-1	819.8	291.6-10252.31	394.9	254.61-650.5	0.0258
MIP-1a	3.06	0-16.88	0	0	0.007
IP-10	6008.66	25.6- 7857.45	22.53	2.51- 592.6	0.0015
Eotaxin	8.70	0-20.29	0	0	0.002
RANTES	0	0	0	0	_

Table 6.3 Levels of cytokines and chemokines found in aqueous humour

	Rejection		Control		
	Median (pg/ml)	Range (pg/ml)	Median (pg/ml)	Range (pg/ml)	p value
IL-2	0	0	0	0	-
IL-4	0	0	0	0	-
IL-6	0	0	0	0	-
IFN-γ	0	0	0	0	-
TNF	0	0	0	0	-
MCP-1	0	0-42.23	19.07	0-47.26	0.41
MIP-1a	0	0	0	0	-
IP-10	53.28	14.48-138.7	45.22	28.2-104.6	0.96
Eotaxin	13.03	0-24.02	25.51	7.06-52.88	0.09
RANTES	4885.61	1866.75-9262.3	4827.2	2171.69-9842.89	0.96

Table 6.4 Levels of cytokines and chemokines found in serum

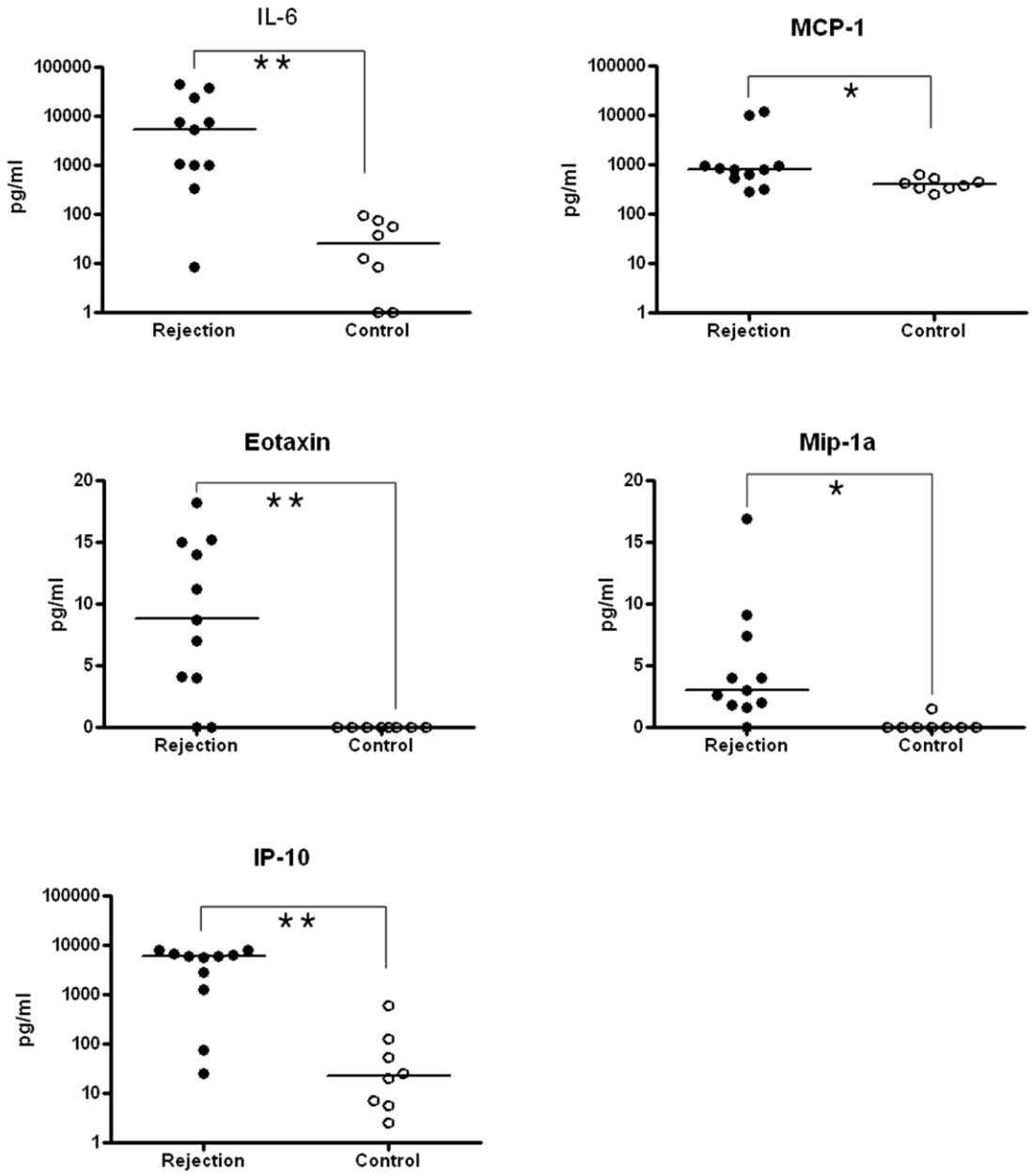


Figure 6.10 Inflammatory cytokines and chemokines

Statistically significant increases in expression of IL-6, MCP-1, IP-10, Eotaxin and Mip-1α were seen in aqueous during rejection compared with controls.

6.4. DISCUSSION

6.4.1. Cells in aqueous humour

Normal aqueous humour is devoid of inflammatory cells, a state maintained by non-permeable capillaries in the iris (the blood-aqueous barrier; BAB) and a contributory factor in ocular immune privilege. Other investigators have reported the absence of leukocytes in aqueous humour samples analysed by flow cytometry(Deschenes et al., 1986,Calder et al., 1999). We also found no leukocytes in normal aqueous. In various types of ocular inflammation the BAB breaks down and inflammatory cells migrate into the aqueous. In corneal graft rejection alloreactive cells which migrate into the aqueous can adhere to and destroy the corneal endothelium: cells can be directly observed at clinical examination as a linear aggregate progressing over a period of days across the donor but not adjacent recipient endothelium.

Although anterior chamber paracentesis is a procedure which carries the risk of intraocular infection, sampling of aqueous humour in anterior uveitis (rather than transplant rejection specifically) for diagnostic and research purposes is relatively common and has been shown to have a low complication rate(Van der and Rothova, 1997). In corneal graft rejection aqueous humour sampling is unnecessary for diagnostic purposes and has rarely been performed for research purposes, presumably due to safety concerns. Although the number of patients recruited to this study was small, no patient suffered an adverse event as a result of aqueous sampling.

With a range of 81 to 837, the number of cells analyzed was low by comparison with typical flow cytometry analyses from other types of sample (i.e. other than aqueous humour). The volume of fluid removed from the eye was necessarily small because the total volume of aqueous is small (approx 600 µl) and thus only a limited amount may be removed without collapsing the anterior chamber and causing further damage to the endothelial monolayer. However, notwithstanding

the small volume of fluid analysed, the cellular yield was low in comparison with aqueous humour samples of patients with acute anterior uveitis. This is probably due to the fact that the anterior chamber reaction in graft rejection is less florid than that in severe anterior uveitis. Investigators of uveitis usually take aqueous samples from eye with the most severe inflammation. Cellular reactions in aqueous of 2+ to 4+ are typical in eyes examined in studies of uveitis(Deschenes et al., 1986) (Wang et al., 1995) . We are confident that despite the low numbers of cells, distinct populations of these 3 cells types could be identified by flow cytometric analysis (see Figure 6.7). However I acknowledge that these results need to be interpreted with caution. In future studies it would be possible to double the yield of cells by not dividing the aqueous sample for staining with isotype-matched controls. Blood cells could arguably be stained with control antibodies.

Our finding of macrophages and lymphocytes in aqueous during acute endothelial rejection is consistent with my data from the mouse model of corneal transplantation and with data from human and animal solid organ grafts(Hancock et al., 1983,Christen et al., 2009). Early experiments by Medawar and Billingham established the adaptive nature of the immune response to allogeneic tissue(Medawar, 1944). These studies coupled with the consistent finding of lymphocytes in acutely rejecting allografts has led to most of the focus, across all fields of transplantation biology, directed at cells of the adaptive immune response and $CD4^+$ lymphocytes in particular. Depletion studies in rodent models of corneal transplantation have demonstrated an important role for $CD4^+$ cells(Yamada et al., 1999a), although recent evidence suggests that rejection may occur independent of these cells(Niederkorn et al., 2006a) . $CD8^+$ cells appear to have the capacity to mediate rejection but their role appears to be less important than $CD4^+$ cells(Yamada et al., 2001). Both $CD4^+$ and $CD8^+$ cells were identified in this study of human aqueous during rejection but at a lower frequency than cells of monocyte / macrophage lineage. The relatively large proportion of $CD14^+$ cells in aqueous humour compared with peripheral blood

suggests selective recruitment of these cells rather than an indiscriminate breakdown of the BAB.

6.4.2. The role of macrophages

Given the relative prominence of $CD14^+$ cells in aqueous humour during rejection, the potential role of macrophages in the rejection process is of particular interest.

There has been recent interest in the potential role of cells of the innate immune response such as macrophages in allograft rejection(Wyburn et al., 2005,LaRosa et al., 2007). Much interest has focused on the early cellular events after transplantation in which innate immune cell infiltration is seen prior to lymphocyte infiltration(Kuffova et al., 2001). Innate immune cells express pattern recognition receptors which discriminate infectious non-self from self, but more recently are also thought to detect host-derived "stress" molecules from damaged tissue. In vascularised grafts ischaemia-reperfusion injury is believed to be a major stressor leading to an innate immune response. While this is less important in transplantation of cornea, an avascular tissue, the mechanical trauma alone of suturing in the donor may cause significant tissue damage. The "danger" model proposed by Matzinger proposes that such alarm signals enhance immunogenicity by activating antigen-presenting cells (APCs)(Matzinger, 2002). As such, the danger model predicts a role of innate immune cells in the afferent limb of the immune response arc and it is very likely that macrophages play an important role in this part of the immune response to allogeneic cornea, one line of supporting evidence being the enhanced rat corneal graft survival reported following depletion of conjunctival macrophages with clodronate liposomes(Slegers et al., 2000). Although we found high proportions of macrophages in aqueous during rejection, we believe that this is unrelated to the surgical trauma response as rejection onset in all patients examined occurred months or years after transplantation.

The reason why acute rejection occurs in some patients up to two or more years after transplantation is unclear. It is possible in some corneal recipients that rejection is precipitated by a sub-clinical allo-independent local inflammatory episode in the cornea, such as infection: in such circumstances macrophages seen in aqueous might represent an innate immune response to this initiating stimulus. Alternatively or in addition, macrophages may constitute part of the efferent immune response to allogeneic tissue. Our finding of macrophages as the highest proportion of cells in an established rejection episode suggests that they have an effector role, at least. Although the presence of macrophages is a consistent finding during rejection of allografts generally, their precise role in the effector phase of the rejection process remains undetermined. Macrophages are among the most versatile of inflammatory cells. Experimental evidence suggests that they are more important in the afferent than in the efferent arm of the immune response to transplanted tissue(Hegde et al., 2005). They are however capable of producing an array of pro-inflammatory cytokines and chemokines, some of which have been shown to be capable of inducing apoptosis of corneal endothelium(Sagoo et al., 2004). Release of cytokines from macrophages into the aqueous is unikely to be a direct cause of endothelial cell loss in human corneal endothelial graft rejection. During rejection endothelial cell loss is limited to the graft with no “collateral damage” seen in host endothelium (which is bathed in the same aqueous) suggesting a more specific method of killing. Two recent reports have demonstrated an important role for macrophages not only in antigen presentation, but as effector cells in the rejection of intraocular tumours(Boonman et al., 2006,Dace et al., 2008). In a mouse model of skin transplantation Yamamoto et al have demonstrated that, allograft-induced macrophages are more cytotoxic than T lymphocytes and that this cytotoxicity is directed specifically towards allogeneic tissue(Yamamoto et al., 1998). While it is known that both $CD4^+$ and $CD8^+$ cells recognise donor cells through MHC class I and class II molecules respectively, the mechanisms by which macrophages might recognise allogeneic cells are unknown. Yamamoto et al postulate the presence

of unique surface molecules distinct from TCR, NK receptor and cytophilic antibodies on allograft-induced macrophages. A recent study by Zecher et al looked at skin hypersensitivity responses to injected allogeneic splenocytes in mice and found that a specific cellular immune response could be mounted against allogeneic cells in lymphocyte-depleted mice(Zecher et al., 2009). This alloreactivity could be reduced by depletion of either neutrophils or macrophages in the host and could be conferred by adoptive transfer of monocytes. Furthermore this response was seen to be dependent on non-MHC disparities between donor and responder strains. These findings also suggest the existence of innate allorecognition systems which are independent of MHC. Horne et al recently described a critical role for effector macrophages in mediating CD4 cell-dependent alloimune injury of transplanted liver parenchymal cells. Horne and co-workers propose a paradigm to explain the specificity of tissue damage by effector macrophages and their results suggest that alloantibody provides the link between the acquired immune response and effector macrophages(Horne et al., 2008). However, on account of the unique immunological features of the anterior chamber microenvironment, findings in rejection of transplants at other sites cannot be assumed to be operational in corneal transplantation. For example Niederkorn and co-workers have specifically examined the roles of macrophages in both afferent and efferent limbs of immune rejection in experimental corneal transplantation and has shown that macrophages are more important in the afferent limb(Hegde et al., 2005).

The CD14 molecule, which has been used to identify macrophages in acute renal graft rejection in humans(Bogman et al., 1989), is a pattern recognition receptor (PRR) which, along with Toll-like receptor 4, acts as a co-receptor for bacterial lipopolysaccharide. Polymorphisms in both CD14 and TLR4 have been shown to influence survival of allografts in humans(Palmer et al., 2007,Palmer et al., 2006). However there is no evidence that PRRs can recognise non-infectious non-self molecules, i.e. alloantigen.

Attempting to pin down the precise role of monocyte or macrophages in an inflammatory process is extremely difficult as the macrophage is an extremely versatile cell in terms of both phenotype and function(Taylor et al., 2005,Gordon and Taylor, 2005).

Macrophages in a tissue may be broadly categorised into those that are resting and those that are activated. Activation may be as part of the innate immune response via pathogen-associated molecular patters (PAMPs) or as part of the acquired immune response either in a “classical” or “alternative” fashion. Classically macrophages are activated by IFN-γ secreted by Th1 $CD4^+$ cells. The activated macrophage secretes its own cytokines and chemokines and phagocytoses antigen. In addition classical activation of macrophages activates the enzyme nitric oxide synthetase (NOS2) which metabolises L-Arginine and leads to release of nitric oxide (NO) and reactive oxygen species. Alternative activation of macrophages is induced by IL-4 from Th2 cells and, in turn, induces an alternative metabolic pathway for L-Arginine by the enzyme Arginase 1 (ARG1)(Gordon and Taylor, 2005). The metabolism of L-Arginine by the enzymes iNOS and ARG in myeloid cells appears to be important in modulating T cell responses(Bronte and Zanovello, 2005).

A population of myeloid cells, known as myeloid suppressor cells, with the capacity to inhibit T cell activation and proliferation have been identified. Filipazzi et al report a population of CD14 monocytes with myeloid suppressor function (even in the absence of NO)(Filipazzi et al., 2007). It is unlikely that the monocyte/ macrophages recruited to the aqueous during cornea graft endothelial rejection are resting. However, even if we assume these cells are activated, we do not know whether their effect is pro- or anti-inflammatory. The absence in aqueous sampkes of the pro-inflammatory cytokines typically associated with graft rejection (IFN-γ and TNF-α) would support a potential suppressive/ modulatory role for the myeloid cells.

Nicholls has reported low levels of both IFN-g and NO in macrophages in anterior chamber in rats undergoing corneal allograft rejection and higher levels of both in

macrophages in the stroma raising the possibility of distinct immunological processes in individual layers of the cornea(Nicholls and Dick, 2008).

6.4.3. Chemokines and cytokines in aqueous during graft rejection

6.4.3.1. MCP-1 and MIP-1α

The expression of MCP-1 and Mip-1α in human aqueous during acute endothelial rejection is consistent with findings in the mouse model of corneal transplantation (Yamagami et al., 1999). In my experiment described in Chapter 3 I compared chemokine expression in whole eyes following transplantation of corneal allografts and isografts respectively. In allografts expression of MCP-1 seemed slightly greater and of MIP-1α seemed slightly less than in isografts. The increased levels of the chemokines MCP-1 and MIP-1α in aqueous were consistent with the finding of large numbers of monocyte / macrophages. MCP-1, which is a ligand of the chemokine receptors CCR1 and CCR5, is chemotactic for macrophages(Charo and Ransohoff, 2006). It is interesting that there appears to be relatively high constitutive expression of this protein in normal aqueous. Funding et al. have also reported constitutive expression of MCP-1 in aqueous with increased expression during rejection and our findings are consistent with this(Funding et al., 2006). The biological role of constitutively expressed MCP-1 remains unknown. Normal aqueous is devoid of leukocytes. However McMenamin has identified a population of macrophages in the iris, which are believed to have a sentinel function as a component of innate immunity(McMenamin et al., 1994). It is possible that constitutively expressed MCP-1 is involved in trafficking of these cells. The increased expression of MIP-1α, whilst statistically significant, was small and is of uncertain biological

significance. Genetic knockout of MIP-1α or its receptor CCR2 in corneal graft recipients did not improve survival in a mouse model of corneal allotransplantation(Hamrah et al., 2007).

6.4.3.2. IP-10

The expression of IP-10 (CXCL10) in human aqueous during acute endothelial rejection is consistent with findings by others in the mouse model of corneal transplantation (Yamagami et al., 1999). I also found greater expression of IP-10 following allografts than isografts in the experiments described in chapter 3. IP-10, a ligand of the chemokine receptor CXCR3, is secreted by monocytes and endothelial cells and is chemotactic for T cells(Baggiolini, 1998). It is possible that expression of these chemokines by macrophages in the aqueous helps to recruit and activate T cells which bear the relevant chemokine receptors(Panzer et al., 2004), in which case these chemokines or their receptors may provide therapeutic targets for the treatment and/or prevention of rejection(Haskova et al., 2007).

The very high levels of IP-10 in aqueous has not been described before but is consistent with findings during rejection of other transplanted tissues(Melter et al., 2001,Segerer et al., 2001). However considerable controversy remains as to its importance and that of its receptor in the rejection process(Halloran and Fairchild, 2008). In particular there appears to be inconsistency regarding the usefulness of CXCR3 and IP-10 as targets to prolong graft survival. Hancock showed that survival of cardiac allografts was improved in CXCR3 KO recipients and in recipients treated with anti-CXCR3 (Hancock et al., 2000). The same group then used anti IP-10 serum and IP-10 KO mice and found that graft survival improved when KO grafts were given to wild type recipients but not when the situation was reversed suggesting that expression of IP-10 from donor cells was more important in the rejection process than in recipient cells(Hancock et al., 2001). More recently there have been conflicting reports which question the

potential of pharmacological blockade of CXCR3 in prolonging graft survival(Kwun et al., 2008,Uppaluri et al., 2008,Zerwes et al., 2008,Rosenblum et al., 2009).
Hamrah found no improvement in survival of corneal grafts in CXCR3- and IP-10 KO recipients respectively(Hamrah et al., 2007).

6.4.3.3. RANTES and Eotaxin

This model and my own results described in chapter 3 also demonstrated increased expression of RANTES during rejection but this was not seen in human aqueous humour. High level constitutive expression of RANTES in peripheral blood was found and this is consistent with one previous report(Whitcomb et al., 2007). Eotaxin, which is chemotactic for eosinophils, was expressed at low levels in human aqueous during rejection and not at all in normal aqueous. Eosinophils were found in rejected corneal grafts in the setting of allergic eye disease in my earlier experiments. In the mouse model I did not find increased expression of eotaxin in allergic eyes during rejection but this may have been due to the timing of the sample. I had hoped that I might recruit some patients with a history of allergic eye disease or atopy for the human study but unfortunately, no patients in our human study had a history of allergic eye disease.

6.4.3.4. IL-6

During endothelial rejection very high aqueous levels of IL-6 were also found. This result is consistent with the findings of Funding et al.(Funding et al., 2005,Funding et al., 2006) IL-6 is a pleiotropic cytokine which is produced by leukocytes (macrophages and T-cells) and by fibroblasts and activated endothelial cells in inflamed tissue(Kishimoto, 2005). It has been identified in aqueous humour in various forms of anterior uveitis(Murray et al.,

1990,Petrinovic-Doresic et al., 1999) and has also been shown to play a role in chronic inflammatory diseases in bowel and joint synovium(Atreya et al., 2000,Nowell et al., 2003). Its first recognised immunological role was as a "B-cell stimulatory factor" which stimulated B lymphocytes to become plasma cells and produce immunoglobulin(Kishimoto, 2005). The receptor for IL-6, IL-6R, is found on not only on leukocytes but also on hepatocytes through which it stimulates production of acute phase proteins such a C-reactive protein.

At the cell surface IL-6 binds to IL-6R and these associate with gp130 a cell surface signal transduction component which is common to several cytokine receptors. IL-6R also exists as a soluble receptor, sIL-6R, and allows this cytokine to influence function of cells not bearing IL-6R. In a process known as trans-signalling IL-6 bind to sIL-6R and these then associate with membrane bound gp130 which is ubiquitously expressed. Trans-signalling in the anterior chamber during acute uveitis appears to be a tightly-regulated process due to increased expression of soluble gp130, an inhibitor of IL-6 trans-signalling(Simon et al., 2008).

A number of specific effects of IL-6 on T-cell function have been described:

It promotes Th2 differentiation(Diehl and Rincon, 2002).

It protects T cells from apoptosis(Teague et al., 1997) and this has been shown to occur in the anterior chamber in acute uveitis(Curnow et al., 2004a).

It inhibits differentiation of regulatory T cells and TGF-β production(Dienz and Rincon, 2009). There is some evidence that IL-6 may reduce ocular immune privilege in anterior uveitis by inhibiting TGF-β(Ohta et al., 2000).

IL-6 also appears to play an important role in the transition from innate to acquired immune response by inhibiting neutrophil accumulation and enhancing recruitment of macrophages and T-cells(Jones, 2005).

The effects of IL-6 on immune cell function are so many and varied that it has been presented at various times as both a pro- and anti-inflammatory mediator(Jones, 2005). The beneficial effects of tocalizumab, a new monoclonal antibody against IL-6R in clinical trials for patients with Crohn's disease(Ito et al., 2004) and rheumatoid arthritis(Jones et al., 2009) provide interesting precedents

for the use of specific IL-6 inhibitors in inflammatory eye disease in the clinical setting. However, in the case of corneal graft rejection, further work using IL-6 KO animals and/or specific inhibitors of IL-6 would be required to try to first unravel the role of IL-6.

6.4.3.5. IFN-γ and TNF-α

IFN-γ is produced by T helper and T cytotoxic cells as well as NK cells. It is the hallmark cytokine of Th1 cells and inhibits Th2 differentiation. It upregulates MHC class I and II expression and activates macrophages(Roitt and Delves, 2003).

TNF- α is produced mostly by macrophages but also by Th cells, B cells and NK cells. It induces E-selectin on endothelium. It activates macrophages and stimulates cytokine secretion(Roitt and Delves, 2003). It also acts with other cytokines to induce features of the “acute phase reaction” such as fever.

Increased expression of IFN-γ and TNF-α has been reported in homogenised rejecting corneal grafts at the mRNA level in the rat model of corneal transplantation(King et al., 2000). Increased TNF expression has been observed at the mRNA and protein levels in rejecting human renal grafts(Krams et al., 1992,Noronha et al., 1992), though the finding of increased expression of IFN-γ in renal grafts has been less consistent(Vandenbroecke et al., 1991,Nast et al., 1994). Rayner measured levels of bioactive TNF in rabbit aqueous humour following corneal allotransplantation. Increased but markedly fluctuating expression of TNF was seen which correlated loosely with the observed onset of endothelial rejection(Rayner et al., 2000). IFN-γ and TNF-α proteins have been detected in human aqueous humour during both corneal graft rejection and anterior uveitis, but at relatively low levels as compared with other cytokines and chemokines(Funding et al., 2006,Sijssens et al., 2007). Sagoo found that prolonged exposure to relatively high concentrations of IFN-γ or TNF- α led to apoptosis of corneal endothelial cells(Sagoo et al., 2004). However immune rejection-induced graft endothelial cell death is unlikely to be caused by

indiscriminate damage to endothelial cells due to increased aqueous levels of inflammatory cytokines. We know this because of the clinical observation that endothelial damage seen in immune rejection is always specific for the graft and leaves the host endothelium, which is bathed in the same aqueous, undamaged. However, given that macrophages and Th1 lymphocytes have been shown to be key mediators of graft destruction and given that these cells were found in human aqueous humour, we might have expected to find their hallmark cytokines in aqueous also. The findings of very low levels or absence of IFN-γ and TNF-α, respectively, in our aqueous samples during graft rejection were not consistent with previous reports and appear counterintuitive.

The reasons for the discrepancies between our findings and those of other transplantation models are not clear. My data suggests that IFN-γ does not play a prominent role in the effector of corneal graft rejection. This is consistent with reports in animal models that genetic knockout of IFN-γ does not alter graft survival in animals mismatched for major and minor histocompatibility antigens(Yamada et al., 2009,Hargrave et al., 2004). Detailed anayses of the rat model of corneal transplantation by Nicholls has shown that IFN-γ is expressed during rejection by cells in the stroma but not by cells aggregating on the endothelium of the graft(Nicholls et al., 2006). Furthermore, on analysis of lymphocytes in the aqueous during corneal allograft rejection in mice, she found few that expressed IFN- γ although, interestingly, many did express TNF-α(Nicholls and Dick, 2008). Taken together these data raise the possibility of differential cytokine expression by cells in different layers of the cornea and in the anterior chamber.

6.4.4. Chapter summary

The work presented in this chapter has demonstrated the following:

- During acute endothelial corneal graft rejection monocytes and helper and cytotoxic T lymphocytes entered the aqueous humour the number of mococytes being relatively higher than those of cytotoxic or helper T lymphoctyes.
- Relatively large increases in expression of IL-6, MCP-1 and IP-10 were seen in aqueous humour during acute endothelial corneal graft rejection while relatively small increase in expression of Eotaxin and MIP-1α were seen.
- No measurable IFN-γ or TNF-α was found in aqueous humour during acute endothelial corneal graft rejection.

In comparison to published histopathology findings on excised rejected human corneal allografts, these reults provide more detailed information on the effector phase of corneal graft rejection and examines much earlier changes. The findings are concordant to a degree with those from my own and othe rodent models of corneal graft rejection but provide further evidence of a significant role of the innate immune system in rejection.

7. Chapter 7: General Discussion of Results

7.1. INTRODUCTION

7.1.1. Corneal transplantation

The cornea is the most commonly transplanted human tissue. 5 year survival rates vary between 90% and 50% depending on the indication for the graft and the presence or absence of other specific features in the host. The commonest reason for graft failure is immune-mediated rejection.

100 years of research into corneal transplantation has provided several key insights into the mechanism(s) of immune rejection. For example, we know that it is a cell-mediated process. However we do not yet have the tools to prevent rejection in every case and particularly in those at high rejection risk.

True immunological tolerance of allografted corneal tissue is the ultimate goal for researchers and clinicians involved in corneal transplantation. This may become a reality in the future. Until then small incremental increases in knowledge about the cellular and molecular mechanisms of rejection may help to identify new strategies to improve graft survival.

7.1.2. Aims of the thesis

My original aims were:

- To investigate the effect of perioperative allergic conjunctivitis on corneal allograft survival and infiltrating cells during graft rejection
- To characterize the phenotype of inflammatory cells in human aqueous during acute corneal allograft endothelial rejection in naïve and atopic recipients of corneal allografts.

Early results from the experiments carried out to address these aims allowed generation of new hypotheses resulting in additional aims. These included:

- To investigate the effect of perioperative allergic conjunctivitis on chemokine expression during graft rejection
- To measure chemokine and cytokine expression in human aqueous during corneal allograft rejection
- To investigate the effect of perioperative allergic conjunctivitis on early post-keratoplasty corneal inflammation and lymphangiogenesis
- To investigate the role of topical dexamethasone 0.1% on corneal lymphangiogenesis and graft rejection in the settinf of allergic conjunctivitis

7.2. ALLERGIC CONJUNCTIVITIS AND CORNEAL TRANSPLANTATION

7.2.1. Summary and discussion of my findings

Prior or perioperative corneal inflammation is a recognized risk factor for corneal graft rejection. The effect of allergic conjunctivitis on corneal graft rejection in humans is unknown but many surgeons treat atopic recipients of corneal allografts with more aggressive topical or systemic immunosuppression on an empirical basis. Mice with allergic mucosal inflammation in either the conjunctiva or airways have been shown to reject corneal allografts at an increased tempo. The reason(s) for this are not entirely clear. A fundamental question is whether this accelerated rejection is due to local effects of allergic conjunctivitis on the cornea and ocular surface or whether it is due to systemic changes in the

immune response brought about by the sensitization protocol which may or may not approximate atopy.

Niederkorn's group has reported results of several experiments with which they tried to answer this question. In these experiments, when allografts were performed in mice with contralateral allergic conjunctivitis the rate of rejection was accelerated in a similar fashion to that in mice with ipsilateral allergic conjunctivitis. Similar results were found in mice with no allergic conjunctivitis but who were exposed to intranasal allergen to produce allergic airway disease. It was concluded from these results that systemic effects of allergy were more important than local effects on corneal graft survival. However they did not study mice that were sensitized to allergen but had no allergen challenge whatsoever (at any mucosal site). Therefore an alternative interpretation of their results might be that allergic inflammation at any mucosal surface (ipsilateral conjunctiva, contralateral conjunctiva, airways) leads to accelerated corneal graft rejection.

In my experiments accelerated rejection was also seen in recipients of corneal allografts with ipsilateral allergic conjunctivitis. There is broad agreement that active ipsilateral allergic conjunctivitis accelerates corneal graft rejection. In relation to this phenomenon, certain aspects of my experiment were novel. The timing of allergen challenge (a single challenge immediately post-operatively) was a new experimental design and showed that perioperative allergic conjunctivitis was sufficient to induce accelerated rejection at a later date. In addition I introduced a novel control group in my experiment: a group of allograft recipients who were sensitized to allergen but not challenged with allergen. These animals did not show an accelerated rate of graft rejection and rejected corneal allografts at a similar tempo to naïve animals. These results suggested to me that local conjunctival allergic inflammation is a more important factor than systemic changes induced by sensitization to allergen in accelerating subsequent corneal graft rejection.

Niederkorn's group felt that systemic effects were most important while my data suggested local factors were more important. It may be that under different

circumstances (eg strain specificity, experimental design) the systemic and local effects of allergy have differential effects on graft rejection. Nevertheless the discordance in the data is unsatisfactory.

The situation is not helped by a lack of clear mechanistic data to support one or other hypothesis. Several associated phenomena have been observed in association with the accelerated rate of graft rejection in allergic conjunctivitis but it is not clear whether these are causal. These include systemic effects such as increased Th2 responses in animals sensitized with allergen. The effect of Th1/Th2 bias on allograft survival has been studied across a wide variety of transplanted tissues, species and experimental conditions and the results have been very inconsistent. Even in corneal transplantation, 2 different groups have shown that Th2-bias *improved* graft survival albeit for MHC-matched grafts only. In my experiment the accelerated rejection of corneal grafts in eyes with allergic conjunctivitis appeared to occur with the hallmarks of Th2-mediated inflammation (eosinophils). This does not offer conclusive proof that systemic Th2 bias accelerates graft rejection. It may be that Th2-bias is merely one of the changes that occur in animals with allergic conjunctivitis and that when rejection is accelerated due to another cause, it occurs via Th2 cells. Sensitized^{+}challenged^{-} animals which have been shown to have increased Th2 responses did not reject their grafts at an accelerated tempo. However if these animals do have increased Th2 responses they are not sufficiently increased to cause infiltration of the graft/ uvea by eosinophils during rejection. It could be therefore that sensitized^{+}challenged^{-} animals have increased Th2 responses but that this Th2 bias is increased even further by challenging with allergen at the time of transplantation leading to subsequent rejection by Th2 cells and eosinophils.

Local changes in the cornea and conjunctiva occur in response to local exposure to allergen and it is plausible that these changes might enhance an immune response to antigen but again there are no conclusive data to prove this. These local responses include:

- Infiltration of the conjunctiva with neutrophils, macrophages, eosinophils and lymphocytes(Choi and Bielory, 2008)
- Alterations in the number and phenotype of conjunctival dendritic cells(Ohbayashi et al., 2007)
- Maturation of corneal antigen-presenting cells(Ozaki et al., 2004)

My experiments have provided new data regarding local corneal and conjunctival changes which occur in corneal transplantation during allergic conjunctivitis and which could accelerate initiation of a specific immune response against alloantigen. Cells of the innate immune system (neutrophils and macrophages) are known to enter the cornea in response to injury as part of the wound-healing response. I have shown that in the presence of allergic conjunctivitis the numbers of these cells entering the host cornea is significantly increased. Macrophages have been shown to play an important role in the generation of new lymphatic vessels which grow centripetally from the corneal limbus following corneal transplantation. My work has shown that in the early days post-transplantaion the ingrowth of these vessels appears to be slightly increased in the presence of allergic conjunctivitis. Furthermore, early post-operative treatment with dexamethasone 0.1% significantly inhibits lymhangiogenesis and mitigates the effects of allergic conjunctivitis on corneal graft survival. On one level these data support a mechanistic role for the increase in corneal lymphangiogenesis in the acclerated rate of graft rejection. However there are at least 2 caveats to this interpretation: 1) dexamethasone 0.1% is not a specific inhibitor of lymphangiogenesis and is known to have many other effects on the immune response and 2) I did not study the effects of dexamethasone treatment on lymphangiogenesis and survival in naïve corneal grafts. We do know that dexamethasone treatment improves survival in the naïve rat model of corneal transplantation. Therefore, although dexamethasone treatment does reverse some of the changes in the graft associated with the presence of allergic conjunctivitis, the improvement in survival seen may be due to the influence of dexamethasone on other factors independent of allergic conjunctivitis.

In the setting of allergic conjunctivitis changes are seen in both the systemic and the local responses to allogeneic cornea. It may be an oversimplification to try to distinguish between the two in terms of their effect on corneal grafts. For example it is possible that active allergic inflammation induces further systemic changes above and beyond those seen in animals who have undergone sensitization alone and that these changes lead to accelerated graft rejection. This would explain the slow tempo of rejection in my sensitized animals which were not challenged as well as the fast rejection in Niederkorns' animals which were challenged at sites other than the eye receiving the graft.
One line of evidence supporting this hypothesis is the widespread systemic breakdown in peripheral tolerance seen following local mast cell degranulation in tolerised skin grafts. Interestingly this phenomenon was seen only for inflammation mediated by mast-cells and not other types of inflammation. Mast cells are known to play an important role in the development of tolerance. Another line of evidence is the presence of eosinophils in the graft / anterior uveal tract of sensitized^{+}challenged^{+} recipients and their absence in sensitized^{+}challenged^{-} recipients suggesting that challenge with allergen at the time of rejection may increase systemic Th2 bias even further. However if this was the case we might expect a similar phenomenon to occur when animals are challenged elsewhere with allergen (eg in the opposite eye or in the lungs). However although challenge at these distal sites leads to accelerated corneal graft rejection, eosinophils are not seen amongst the graft infiltrating cells.

If local conjunctival changes alone are responsible for the accelerated rate of rejection in allergic conjunctivitis then the question arises as to whether this would be true of conjunctivitis secondary to any other cause. We did not study this question but it is an important one. We are not aware of any other model of conjunctivitis which is as consistent, as florid and yet humane as the allergic conjunctivitis model. I think that it is likely that local changes such as changes in APC number and function and even lymphangiogensis would occur with

conjunctivitis of any cause and would not be surprised to see acceleration of graft rejection in the presence of florid conjunctivitis of any cause. However if the fate of the grafts is influenced also/ instead specifically by mast cell cell degranulation then other types of conjunctivitis may not have as significant an effect on graft survival as allergic conjunctivitis.

When considering the clinical implications of these experiments, the first thing to consider is what the model actually represents in human terms. I feel that of all the types of allergic conjunctivitis seen in humans, this animal model is most akin to seasonal allergic conjunctivitis in that it is caused by IgE-dependant mast cell degranulation(Fukuda et al., 2009). Therefore, on the most fundamental level, these results suggest that one should not perform a graft in a patient with active SAC. The local effects of SAC are self limiting and are usually modifiable with topical treatment so keratoplasty should be postponed until conjunctival disease is quiescent. Considering more chronic forms of allergic conjunctivitis such as AKC we are probably moving away from what the model represents which makes it less easy to know how to translate the findings to a human clinical scenario. In his experiments Beauregard continued to challenge his mice regularly with allergen following corneal transplantation (as compared to my single postoperative challenge) and he observed accelerated graft ejection in these eyes. Chronic exposure of the conjunctiva to allergen produces a more chronic form of allergic conjunctivitis with local expansion of conjunctiva-associate lymphoid tissue (personal communication Mark Ohbayashi). Therefore it is probably safe to extrapolate my findings to include more chronic forms of allergic conjunctivitis. Again best possible control of local conjunctival inflammation before performing keratoplasty would be ideal. AKC can be more difficult to control than SAC but this disease usually responds to topical corticosteroid of appropriate potency, frequency of instillation and length of treatment. Standard treatment following penetrating keratoplasty involves intensive instillation of topical corticosteroid in the first post-operative weeks with gradual tapering of the

frequency of instillation over subsequent months. In patients at high-rejection risk the frequency of instillation is tapered more slowly/ over a longer period.

The one thing we can not control is the patients underlying tendency toward atopy. The effect of atopy on graft survival remains a grey area. Although my results suggest that this is less important than the local activity of the disease in the eye in its influence on graft survival, there are other lines of investigation which refute this. Niederkorn's group have reported accelerated corneal graft rejection in recipients with both allergic conjunctivitis and allergic airways disease and attributed this to the systemic changes in immune response seen in these atopic conditions(Niederkorn et al., 2009,Beauregard et al., 2005). Cursiefen has reported a reduction of human graft survival in recipients with atopic dermatitis with or without ocular involvement(Nguyen et al., 2008). Yildiz et al have reported a tendency towards higher rates of graft rejection in patients with a self-reported history of atopy(Yildiz et al., 2009). Ironically, the limited data available on graft recipients with allergic eye disease (VKC) suggests that outcomes are reasonably good in terms of graft survival(Wagoner and Ba-Abbad, 2009) but as mentioned in Chapter 1, studies on human allergic conjunctivitis are open to several errors such as low power ,loose phenotyping and variable post-operative treatment regimes.

Based on the findings by myself and others as described above I would consider the following patients as increased rejection risk:

- Active conjunctivitis of any cause
- History of AKC or VKC whether active or not
- History of atopy

Post-operatively I would treat all alloantigen-independent inflammation of the ocular surface aggressively.

7.2.2. Future work

There are two ways of looking at the future in relation to my work in this area. Firstly I see experiments which could be done to address issues which arose directly from my own work. These include:

- Challenging sensitized mice at various timepoints post-transplantation to study the effect of the timing of challenge on graft survival.
- Transplantation in mice with non-allergic conjunctivitis to assess the specificity of the change in tempo of graft rejection seen in allergic conjunctivitis.
- Treatment of recipients with mast-cell stabilizer prior to transplantation/ challenge to properly assess the effect of mast-cell stabilistation.
- Fitting of mice with anti-scratch collars to prevent eye-rubbing after challenge to assess the possibility that eye-rubbing might contribute to the accelerated rate of graft rejection in allergic conjunctivitis.

In relation to the (slightly) earlier post-keratoplaty corneal lymphangiogenesis seen in eyes with allergic conjunctivitis, I would have liked to carry out the following further experiments:

- Measurement of the effect of topical dexamethasone treatment on corneal lymphangiogenesis and graft survival in naïve eyes.
- Measurement and comparison of donor-induced DTH responses (ear swelling assay) at 1 and 2 weeks in naïve and allergic recipients of corneal grafts, the hypothesis being that eyes with earlier lymphatic ingrowth would be sensitized earlier and show a more marked DTH response at 1 week.
- Measurement and comparison of VEGF A and VEGF C expression in the cornea of naïve and allergic recipients of corneal allografts at days 2 and 6.
- Measurement of the effect of neutrophil depletion on corneal lymphangiogensis and graft survival in naïve and allergic animals.

- Analysis of the effects of allergen exposure on conjunctival lymphatic vasculature using immunohistochemistry and wholemounted tissue. A former laboratory colleague has begun this project and his early work has demonstrated a marked increase in both lymphoid tissue and associated lymphatic vessels I the conjunctiva in a model of chronic allergic conjunctivitis involving repeated exposure to allergen.

Other experiments while not related directly to issues arising from my own work will help provide important data concerning the effects of atopy and allergic conjunctivitis on the immune response to donor cornea. UK transplant collects demographic and sequential clinical data including history of atopy on all corneal graft recipients in the UK. Future comparison of survival data and incidence of rejection between atopic and non-atopic patients will provide further evidence to help answer the question of whether atopy is a significant risk factor for human corneal graft rejection.

As long as the causes of atopy remain unknown it will be difficult to untangle its immunological sequelae. Advances in understanding of pathophysiology of atopy will help to understand its effect on individual tissues.

A more definite area for future investigation is that of inhibition of corneal lymphangiogenesis. Inhibitors of VEGF A have been shown to be effective at inhibiting corneal lymphangiogensis in animal models and at improving corneal graft survival(Bachmann et al., 2008,Cursiefen et al., 2004a). However further work is required to tease out the differential benefits of inhibition of haemangiogenesis and lymphangiogenesis. Pilot studies in animals and humans have shown that topical use of the anti-VEGF A monoclonal antibody (mAb) bevacizumab is safe and effective at reducing established corneal vascularistation(Dastjerdi et al., 2009a). In a mouse model of high-risk corneal transplantation due to vascularisation of the host cornea, subconjunctival but not topical bevacizumab improved graft survival(Dastjerdi et al., 2009b). Bourghardt Peebo et al have recently identified lymphatic vessels in vivo in human cornea using confocal microscopy(Bourghardt et al., 2009). Using these tools it will be

possible to study the effects of topical anti-VEGF mAbs on post-keratoplasty corneal haemangiogenesis and lymphangiogenesis in humans.

It is clear that angiogenesis is caused by allo-antigen independent inflammation in the cornea(Cursiefen et al., 2004a). It is impossible to perform graft without causing allo-antigen independent inflammation but the challenge is to minimize it at the time of transplantation and thereafter. Despite advances in technology one of the commonest sources of alloantigen-independent corneal inflammation in humans is the sutures used to secure the graft. A broken suture in particular serves as a marked stimulus to local inflammation. But even adequately tight/ buried sutures provide a stimulus for local corneal angiogenesis and lymphangiogenesis. New femtosecond laser technologies are providing novel ways to cut donor and recipient corneas so that the donor and recipient material "lock" together more securely than the traditional hand-cut graft and so may require fewer sutures. Whether this reduces corneal angiogenesis & lymphangiogeneis remains to be seen.

7.3. CELLULAR MEDIATORS OF CORNEAL GRAFT REJECTION

7.3.1. Summary and discussion of my findings

The precise mechanism(s) by which cell death is brought about during immune rejection of transplanted corneal tissue remain unknown. Experiments on laboratory animals have shown that immune rejection of transplanted tissue is an acquired immune response that is a) specific and b) cell-mediated(Medawar, 1944,Mitchison, 1954). Direct observation of corneal endothelium during rejection reveals aggregates of immune cells adhering to the transplanted endothelial cells but not the host endothelial cells. Often the cellular aggregates form a line which slowly "marches" across the grafted endothelial sheet leaving dead or dysfunctional endothelial cells in its wake. The precise make up of these cellular aggregates and the precise method by which they induce endothelial cell death remain uncertain.

Histopathological studies of human and animal rejected grafts have described consistently $CD4^+$, $CD8^+$ and macrophage cell populations. Other cell types such as NK cells and eosinophils have been found but with less frequency. Human pathological studies are limited by the fact that what is seen on the histopathology may not represent what is happening during acute rejection as human grafts are usually replaced at a long interval after graft failure. Which cell(s) are most important in graft rejection? Experiments using gene KO animals and depleting antibodies suggest that CD4 cells are the most important in that they are necessary for graft rejection to occur. (Niederkorn has suggests that even these are not necessary) However we do not know whether the CD4 cell is capable of inducing cell death directly itself or whether it does so by directing the activities of other cells like CD8 or macrophages.

My data confirm previous reports of $CD4^+$, $CD8^+$ and macrophage cell populations infiltrating mouse corneal grafts during rejection. I have also performed flow cytometry of human aqueous during corneal graft rejection and reported the presence of $CD45^+$ cells therein. Control aqueous from normal eyes

did not contain $CD45^+$ cells. I have further identified and quantified $CD4^+$, $CD8^+$ and $CD14^+$ subsets of these leukocytes in human aqueous during endothelial graft rejection. While the numbers of cells were admittedly quite small and the results do need to be interpreted with some caution, there did appear to be more macrophages in human aqueous during rejection than either $CD4^+$ lymphocytes or $CD8^+$ lymphocytes. Questions which remain to be answered about monocytes in the anterior chamber during graft rejection include:

- Is their net effect pro-or anti-inflammatory?
- Do they contribute directly to endothelial cell death? If so, how?
- Do they contribute indirectly to endothelial cell death? If so, how?

In the mouse corneal graft recipients with active perioperative allergic conjunctivitis eosinophils were seen amongst the graft infiltrating cells during rejection.

7.3.2. Future developments

The limitation in terms of further flow cytometric studies on human aqueous in graft rejection is the low cellular yield from aqueous samples. This could be improved by not splitting the aqueous sample for staining with isotype-matched controls (blood cells could arguably be used instead for control analysis). It may also be improved by selection of patients with the most severe clinically observable anterior chamber reaction for sampling and possibly by refining the staining technique. The flow cytometry experiments I would like to run include:

- Identification and quantification of NK cells in aqueous
- Identification and quantification of the regulatory T cell subset of CD4 cells
- Measurement of costimulatory molecules and reciprocal receptors on monocytes and lymphcytes.
- Measurement of arginase /iNOS in monocytes.

Intravital microscopy/ live imaging of immune cells is a relatively new discipline which is providing new and important information on immune cell structure and function(Spencer et al., 2008). In vivo and ex vivo studies using confocal

microscopy and two-photon fluorescent microscopy provide 3D and 4D (x,y,z and time) data on immune cells and their movements and interactions with other cells(Nitschke et al., 2008). Confocal microscopy has been used to image dynamic interaction of leukocytes with vascular endothelium in human allergic conjunctivitis.(Lim et al., 2006) Using confocal microscopy we have been able to image leukocytes adherent to the endothelium during acute endothelial rejection. We are aware from our flow cytometry study that there are at least 2 types of immune cell in the anterior chamber during rejection –lymphocytes and monocytes. Analysis of confocal microscopy images in human cornea relies on morphology to distinguish cell types but in the images we generated of corneal endothelium I could not identify any clear morphological features which could be used to identify these two cell types. Two-photon fluorescence microscopy has allowed identification and dynamic imaging of individual cell subtypes in experimental animals(Beltman et al., 2009). Cells are marked either by genetic manipulation of the animal so that the cells of interest express green fluorescent protein or by reconstitution of animals with fluorochrome-labelled cells or both. Using this technology, investigators have measured changes in dendritic cell morphology and motility in response to antigenic and other stimuli in bowel, skin and cornea(Ward et al., 2007,Nishibu et al., 2006,Chieppa et al., 2006). Valuable information about T cell activation in lymph nodes has also been obtained by studying movies of APC/lymphocyte interaction therein(Bousso, 2008). There are fewer reports of two-photon imaging of effector cell dynamics in inflamed tissue but cornea, being transparent, would be a tissue most suited to such studies.

7.4. CYTOKINE AND CHEMOKINE EXPRESSION DURING CORNEAL GRAFT REJECTION

7.4.1. Summary and discussion of my findings

I studied chemokine expression around the time of corneal graft rejection in humans and mice. My results are summarized in Table 7.1.

	Mouse	Human
IP-10	+	++
RANTES	+	-
MCP-1	-	++
MIP-1α	-	+
Eotaxin	-	+
MIP-1β	-	Not tested
Lymphotactin	+	Not tested
TCA-3	-	Not tested
MIP-2	-	Not tested
IL-2	Not tested	-
IL-4	Not tested	-
IL-6	Not tested	++
IFN-γ	Not tested	-
TNF-α	Not tested	-

Table 7.1 Chemokine and cytokine expression during corneal allograft rejection.

Expression was measured in whole mouse eyes using ribonuclease protection assay and in human aqueous humour using cytometric bead array.

IP-10 was expressed in both mouse and human eyes during rejection. Other results were less consistent. These differences may reflect species-specificity of the response but there were also fundamental differences in the study designs which may account for these differences. The mouse samples were taken at a

fixed timepoint which may or may not have been the point of maximal chemokine expression. The study in mice used a less sensitive assay (RPA) than that in humans (CBA) and the study in mice measured RNA in whole eyes whereas the human study measured protein expression in aqueous humour.

In mice there was no evidence of differential expression of chemokines in naïve and allergic recipients of corneal grafts. The finding of increased expression of IP-10, RANTES and Lymphotactin was consistent with previous reports(Yamagami et al., 1999). The finding of increased IL-6 and MCP-1 in human aqueous was also consistent with a previous report(Funding et al., 2006) but the presence of IP-10, Eotaxin and MIP-1α has not been previously reported.

Animal studies using gene-knockout animals and specific chemokine/ cytokine and chemokine/ cytokine receptor inhibitors can provide information as to the specific role, if any, of individual chemokines, cytokines and their receptors in an inflammatory process as well as identifying potential therapeutic targets. Some of these studies have already been carried out in relation to corneal transplantation the results of which are summarized in Table 7.2.

Intervention	**Reported effect on allograft survival**
IL-1R antagonist	Significantly prolonged(Dana et al., 1997)
IFN-γ KO host	No effect(Yamada et al., 2009)
CCR1 KO host	Significantly prolonged(Hamrah et al., 2007)
CCR2/MIP1α KO	No effect(Hamrah et al., 2007)
CCR5 KO host	No effect(Hamrah et al., 2007)
CXCR3 KO host	No effect(Hamrah et al., 2007)
Anti-RANTES ab	No effect(Hamrah et al., 2007)
MIP1α KO host	No effect(Hamrah et al., 2007)
IP-10 KO host	No effect(Hamrah et al., 2007)
Anti-KC	Significantly prolonged in High-risk model(Amescua et al., 2008)

Table 7.2 Reported effect of targetting cytokines/ chemokine and their receptors on corneal allograft survival

7.4.2. Future Work

Whilst I did not find any evidence of differential chemokine expression in naïve and allergic recipients of corneal allografts, my study design was such that I cannot say conclusively that there is no difference in chemokine expression. A future study measuring chemokine expression at sequential timepoints following transplantation in these two groups may provide a more conclusive answer to this question.

Future animal studies using gene-knockout animals and specific chemokine and chemokine receptor inhibitors will provide information as to the specific role if any of individual chemokines, cytokines and their receptors in corneal graft rejection. Monoclonal antibodies against cytokines/chemokines or their receptors are typically licensed for use in common chronic inflammatory conditions such as rheumatoid arthritis and inflammatory bowel disease and then, based on research which demonstrates common cellular/ molecular pathways of inflammation, are used off-licence to treat ocular inflammatory conditions. This has been the case for anti-TNF monoclonal antibodies in uveitis(Vazquez-Cobian et al., 2006). Many more monoclonal antibodies are emerging for clinical use in the treatment of inflammatory conditions eg Tocilizumab (mAb against IL-6R) in rheumatoid arthritis(Jones et al., 2009). Several companies are carrying out Phase 2 clinical trial of small molecule inhibitors or the chemokine receptors CCR1, CCR2, CCR5 and CXCR3(Charo and Ransohoff, 2006).

Given the acute nature of graft rejection it is difficult to imagine mAbs against cytokines, chemokines and their receptors being generally useful as treatments of acute rejection in the way that they have been in more chronic forms of ocular inflammation. But it is possible that as we gain more information about their specific roles in the rejection process, they may be of use in prevention of rejection in certain patients at higher risk of rejection in future.

7.5. PUBLICATIONS ARISING FROM THE WORK PRESENTED IN THIS THESIS

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In: Darlene A. Dartt, Editor. Encyclopaedia of the eye, Vol 3. Oxford: Academic Press; 2010. pp. 290-295

The Effect of Allergic Conjunctivital Inflammation on the Allogeneic Response to Donor Cornea
Flynn TH, Ohbayashi M, Ikeda Y, Ono SJ, Larkin DF
Investigative Ophthalmology and Visual Science 2007; 48(9): 4044-9

Aqueous humour alloreactive cell phenotypes, cytokines and chemokines in corneal allograft rejection
Flynn TH, Mitchison NA, Ono SJ, Larkin DF
American Journal of Transplantation 2008; 8(7): 1537-44

Use of ultrasonic pachymetry for measurement of changes in corneal thickness in mouse corneal transplantation
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