Studies of RPE as an immune privileged tissue and as the creator of immune privilege in the subretinal space.

Parisa Zamiri MBBS FRCOphth

A Thesis for the Degree of

Doctor of Philosophy

The Institute of Ophthalmology

University College London
To the memory of my
Grandmother Halime Dianaty
who was blind

and to

Mike Dobson, my Biology Teacher, who instilled in me the love for science
In Memoriam

J. Wayne Streilein MD
19th June 1935
15th March 2004

If I should die and leave you here awhile,
Be not like others, sore undone, who keep
Long vigil by the silent dust and weep.

For my sake turn again to life and smile,
Nerving thy life and trembling hand to do
That which comforts other souls than thine;

Complete these dear unfinished tasks of mine,
And I, perchance, may therein comfort you.

Mary Lee Hall.
Acknowledgements

Throughout life one is constantly redefined with every major experience. At no time in my life I have felt this to be truer than during the past few years as I have been learning the art of hypothesising, performing experiments, and re-evaluating my thoughts. I started this PhD with a naïve thought of making a small mark in the knowledge of mankind. Instead it changed my life.

In many ways the process that begins in enrolment for a Ph.D. and ends in writing a thesis is similar to being born as a child and raised to adulthood. They say, “It takes a village to raise a child”. A large village was involved in completion of this thesis.

Dr. Streilein held my hand every step of the way. From him I learnt humility, steadfastness and the love of science. He taught me the joy of a new discovery and enabled me to withstand the pain of defeat. He has an amazing ability to elevate people above the ordinary. I feel a better person in his presence. Sue Lightman gave me the gift of knowing what is important in life and the courage to stand outside the box. Without her encouragement, advice and support I would not have commenced on this journey.

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During the past few years many people were added to the village that raised me. Tanja Sleger with her quiet and sincere presence has left a lasting impression. I miss you Tanja. Steven Ijland taught me courage by following his ideas and dreams. Right on, Steven! Simon and Helen Whiteley provided much needed sense of humour and taught us to play Bridge. Simon and Helen together with Rebecca Roth, Jennifer Ross-Chen, Amy Warshawski and Ruby Ghaffari have shared their time, love, food and expertise in child rearing with us. We have formed a friendship and help network to support each other through first few years of motherhood. Manuel and Carol Cecerrano cared for my children and I as surrogate parents.
They allowed me the flexibility of time needed to be in the lab. I always had difficulty
dragging my kids away from their loving arms.

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my life but they surpassed themselves in the past few years. My mother selflessly looked after
my children and my home far away from her own life making sacrifices all along. My father
taught me that I can do anything in life, move the mountain if needs be. He lived alone
without his wife for much of this time. My debt to them is immense.

The biggest miracles of my life, my children Paria Elisabeth (Gugulia) and Aram
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given me purpose. They put problems in perspective and a hug from their tiny arms around
my neck made all the worries disappear.

My husband, Christian Reich, taught me to have confidence in my abilities. He
challenged me to rethink my ideas, to explain my hypotheses from the first principal and
above all to never be afraid to fight for what I believe in. We have worked as a team on this
thesis, organising, formatting, thinking, screaming and laughing. This thesis, is every bit as
much his as it is mine.
Abstract

The aims of this thesis were to investigate the role of the retinal pigment epithelium (RPE) in immune privilege of the subretinal space, and to determine the vulnerability of RPE to immune-mediated attack. An ex-vivo model of the RPE eyecup was formed from adult C57BL/6 with /without prior treatment with sodium iodate (SI), as well as thrombospondin-1 knockout (TSP-1KO) and rd mice, by removing the anterior segment and retina and leaving an intact monolayer of RPE together with choroid and sclera. The supernatant (SN) collected from the RPE eyecups which were cultured with serum free medium were used in T cell proliferation, ELISA and cytotoxicity assays to determine the contribution of RPE to the putative immunosuppressive microenvironment of the subretinal space. In addition, the role of RPE in the presence of immune deviation, and in response to experimental autoimmune uveoretinitis was assessed.

RPE in the eyecup preparation was relatively resistant to immune mediated attack by allo-sensitised T cells. RPE SN contained both latent and active TGF-β and thrombospondin, required for TGF-β activation. RPE SN also contained significant amount of somatostatin (SOM), a neuropeptide. RPE SN profoundly inhibited T cell proliferation and IFN-γ production by antigen and anti-CD3 stimulated T cells in an active TGF-β and SOM dependent manner. Moreover, SN of RPE eyecup significantly inhibited IL-1β production by activated neutrophils and macrophages and diminished NK cells lysis of YAC-1 target cells. SN of RPE eyecups from SI- treated, or TSP-1KO and rd mice were deficient in their capacity to create an immunosuppressive microenvironment and lacked immune deviation to OVA antigen injected into their subretinal space. TSP-1 KO mice, unlike wild type mice, suffered a prolonged and severe uveoretinitis. These results indicate that RPE possess inherent immune privilege and acts as the master organiser of immune privilege in the subretinal space by producing immunosuppressive factors.
Credits

Mr. Peter Mallen produced Figure 1.1, a masterful representation of the retina and the subretinal space.

Dr. Sharmila Masli performed PCR analysis for thrombospondin and somatostatin.
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FITC  Fluorescein Isothiocyanate
GAG  Glycosaminoglycan
GH  Growth Hormone
GM-CSF  Granulocyte-Macrophage-Colony-Stimulating Factor
H&E  Hematoxylin and Eosin
HBSS  Hank’s Balanced Salt Solution
HCl  Hydrochloric Acid
HFRPE  Human Foetal Retinal Pigment Epithelium
ICAM-1  Intercellular Adhesion Molecule-1
I/CB  Iris/Ciliary Body
ICE  IL-1β Converting Enzyme
i-EIU  Local endotoxin-induced uveitis
IFN-γ  Interferon-γ
Ig  Immunoglobulin
IGF  Insulin-like Growth Factor
IL  Interleukin
IL-1α  Interleukin-1-α
IL-1β  Interleukin-1-β
IL-1RA  IL-1 Receptor Antagonist
IL-2  Interleukin-2
IL-6  Interleukin-6
IL-8  Interleukin-8
IPE  Iris Pigment Epithelium
LAP  Latency Associated Peptide
LC  Langerhan Cells
LPS  Lipopolysaccharide
LTBP  Latency Associated Peptide
MAIU  Mycobacterial Adjuvant-Induced Uveitis
MCP  Monocyte Chemotactic Protein
M-CSF  Macrophage-Colony-Stimulating Factor
MHC  Major Histocompatibility Complex
MIF  Macrophage inhibitory Factor
ml  Millilitre
μl  Microlitre
mM  Millimolar
mRNA  Messenger Ribonucleic Acid
NK  Natural Killer
NKT  Natural Killer T cells
nm  Nanometer
NO  Nitric Oxide
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<td>TNFRp75</td>
<td>Tumour Necrosis Factor-Receptor p75</td>
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<td>TSP-1</td>
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<td>TSP-1KO</td>
<td>Thrombospondin-1 Knock-Out</td>
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<td>UPA</td>
<td>Urokinase-type plasminogen activator</td>
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<td>VCAM-1</td>
<td>Vascular Adhesion Molecule-1</td>
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<td>VEGF</td>
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<td>Vasoactive Intestinal Peptide</td>
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<td>WHO</td>
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<td>ZO-1</td>
<td>Zonular Occlusion-1</td>
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Chapter 1 – Introduction

1.1 Background

So much of the information we glean from the world around us is gathered through our eyes. Eyes are exalted in literature of the world and have been likened to the window of our souls. We express our emotions through our eyes, choose a mate, work and take pleasure. From ancient times to the renaissance and beyond, blindness has been depicted in art and its sufferers were both pitied and despised, lest they transfer their curse onto the onlookers. With few exceptions in history, the most that the blind could hope for was to be successful beggars. One of the famous exceptions, the poet Homer, was said to receive the gift of genius in place of his sight. Treatment of blindness was looked on as a miracle performed by Jesus (Holy Bible, Mark 10:46-52). As early as 5000 BC, the earliest medical record known to us, the ancient river cultures of Mesopotamia had ophthalmology as a medical specialty.

Views of blindness and the role of the blind in society have changed. Blindness, however, remains as the most devastating misfortune of a person’s life. According to the world health organisation (WHO), 38 million people are estimated to be blind and a further 110 million have low vision and at the risk of becoming blind. As well as individual suffering, blindness leads to loss of productivity and income and requires rehabilitative and supportive care. The economic burden of blindness worldwide is enormous. The cost of blindness in the USA was put at $4.6 billion dollars in 1994 and in India at $4.1 billion dollars per year (WHO).

In the developed world, effective treatments are available for many potential causes of blindness such as cataract and corneal diseases. The most common causes of blindness in the industrial world are diseases affecting the retina, such as diabetes and diabetic retinopathy, and hereditary retinal diseases in younger age groups as well as age related macular degeneration in the older population. Although some preventive measures are available in the case of diabetic retinopathy, no satisfactory treatment exists for retinal diseases that lead to blindness.

There has been increasing interest in the last decade in orthotopic retinal transplantation as a promising tool of restoring vision to eyes blinded by destructive retinal
disease. The retinal photoreceptors together with retinal pigment epithelium (RPE) form a functional unit. Any environmental or genetic change in the delicate balance of this unit leads to loss of photoreceptors as well as RPE. Since photoreceptors are unable to regenerate in mammals, the consequence is a progressive visual deterioration and eventual blindness. Age related macular degeneration (AMD) and retinitis pigmentosa (RP) are amongst a spectrum of diseases that fall into this pattern and for which retinal transplantation may offer hope.

1.2 Age related macular degeneration

Age related Macular degeneration (AMD) is the leading cause of registered blindness in the developed world. Approximately 30% of people over 75 suffer from one form of this devastating disease. In cross sectional population studies, about 45% of eyes with AMD have visual acuity reduced to 20/200 or worse. People suffering with AMD are frequently in need of assistance in reading, driving and eventually performing their basic needs. With an increasingly aging population, both the incidence and prevalence of the disease will rise.

There are two distinct clinical manifestation of AMD: Geographical atrophy ("dry") AMD and the exudative ("wet") variety. The relative incidences of wet and dry forms of AMD are varied amongst different studies depending on the criteria used to define geographic atrophy (Penfold 2001). Ferris et al included drusen together with atrophic lesion in their definition of dry AMD (Ferris 1983). Comparing the incidences of pure geographic atrophy with neovascular AMD, several investigators found that exudative AMD were twice as common as the geographical atrophy (Mitchel 1995, Green 1993).

Although there is a genetic component to AMD, the study of heritable causes of AMD is complicated due to diverse phenotypes and late onset of disease (Chung 2002). Recently in one large family with AMD, an AMD locus has been identified on chromosome 1q25-q31 (Klein 1998). Histopathological studies indicate that there is a loss RPE component of blood retinal barrier (BRB) at the macula in atrophic AMD (Penfold 2001). Neovascular AMD primarily affects the choriocapillaries and secondarily the RPE.

Drusen, focal deposits of extracellular material that form between the RPE and Bruch’s membrane, are significant risk factors for development of AMD. Drusen are loosely divided into morphologically distinct groups of “small, hard” drusen and “soft, confluent” drusen. Although many studies attempt to correlate the shape and size of drusen to the
development of the eventual disease phenotype, only soft, large confluent drusen have been shown to correlate with development of choroidal neovascularisation. Drusen are also associated with other ocular conditions such as long standing retinal detachment, over malignant melanomas (Fishman 1975) and pigmented nevi (Naumann 1966) as well as in familial macular degenerations such as Sorsby's fundus dystrophy (Polkinghorne 1989).

There is a growing belief that there is an immune pathology associated with AMD. Part of the evidence comes from the presence of drusen in membranoproliferative glomerulonephritis type II (D'Souza 2000, Duvall-young 1989), which are morphologically and compositionally similar to those found in AMD. The components of drusen include apolipoprotein E, factor X, amyloid A, vitronectin, immunoglobulin lambda chains, activated complement components including C5b-9 complex and MHC class II antigens (Johnson 2000, Mullins 2000, Mullins 2001) all of which are in some way associated with immune system (Hageman 2001). All of these can be synthesised locally by RPE, retinal and choroidal cells. Other investigators have noted accumulations of giant multinucleated cells (Penfold 1985) and other leukocytes (Penfold 1985 and Killingsworth 1990) in the choroids of donors with AMD. Looking at the above evidence it is reasonable to suggest that there is immunopathological aetiology to AMD.

1.3 Retinitis pigmentosa

The term retinitis pigmentosa (RP) encompasses a large disparate group of genetic diseases leading to progressive photoreceptor regeneration and hence visual impairment. The prevalence of the disease worldwide is about 1.5 million, which is equivalent to 1 in 3000-4000 individuals. In the western world after diabetic retinopathy, RP is the most common cause of blindness in people under the age of 70. The clinical pictures that have led to RP family of diseases sharing a common group are based on symptoms, clinical examination, genetics and electroretinographic studies.

Clinically there are pigmentary changes resulting from release of pigment from degenerating retinal pigment epithelial cells that migrate to the neural retina and accumulate in the perivascular clusters known as “bone-spicule”. Atrophy of the retina leads to attenuation of the blood vessels providing a typical appearance on fundal examinations. In the most common cases of RP, the rods are predominantly affected leading to the characteristic night blindness, loss of mid peripheral fields and early age of onset of symptoms. If the cones
are equally affected, the patients will in addition to the above symptoms, also suffer from a more central loss of the visual field.

Historically the name “retinitis” was coined, as there was a belief that the patients suffered from retinal inflammation (Acsadi 1991). It is now universally believed that RP is a genetically determined disease with many different genotypes and even more diverse phenotypes. However there is ample evidence that the immune system plays a role if not in initiation of the disease process but in subsequent course, and may play a part in exacerbating the condition (Kaplan 1999b). The age of the onset of the disease varies from infancy to the middle age depending on the mode of inheritance with the x-linked RP starting the youngest, followed by autosomal recessive and dominant inheritance.

Research into RP has partially concentrated on prenatal diagnosis of the disease and prognosis of visual outcome to the affected individuals. Emergence of recombinant DNA technology has led to the possibility of detecting molecular defects by using any tissue that contains nucleated cells. In future, it may be possible to use this technology to find closely linked DNA markers near any gene of interest leading to prenatal or preclinical diagnosis (Sharma 1999). At present, prenatal diagnosis of isolated cases is impossible and that of recessive forms are very limited. Multiple loci are often linked with very similar clinical appearance. For example, eight separate loci have been identified in Usher syndrome by linkage studies (Chaib 1997). Electroretinographic data have enabled investigators to partially identify carriers of choroideraemia and x-linked RP. The character of the underlying molecular defect has correlated well with the clinical outcome: A point mutation of the proline in codon 347 to either leucine, serine or arginine leads to a more severe clinical picture (Dryja 1990, Gal 1991), whereas most patients with mutations at codon 23 of rhodopsin with a proline to histamine substitution present with a regional, less severe form of RP (Oh 2003). The problem of prognostic counselling, however, is confounded by the fact that the same genetic defect may be present within one family with multiple phenotypes.

1.4 Treatment modalities for retinal degenerations

At present there are no effective treatments for either of these disease entities, but there are promising inroads into potential approaches to treating retinal degeneration. As photoreceptors are unable to regenerate, the approach to treatment should aim to prevent photoreceptor loss or replace lost photoreceptors. Treatments directed at prevention of loss
include diet modification and drug therapy, growth factor administration, gene therapy, and cell transplantation (Lund 2001). Treatments aimed at replacing lost photoreceptors include cell transplantation and microelectrode prosthesis placement (Lund 2001). In addition, many surgical procedures have aimed at replacing the diseased macular area in AMD by transposition of a healthy part of the retina to the macular area.

1.5 Current treatment modalities

Most current modalities of treatment have aimed at prevention of visual loss by medical treatment (see below), controlling the complication of retinal diseases as well as supporting the patients who have become blind. Since excess light has been known to increase the rate of photoreceptor loss in experimental animals (Naash 1996, Wang 1997), reducing light exposure is suggested, though clinical support of these findings is still unclear (Sharma 1999). Visual aids and optical devices such as wide-angle optical systems that compensate for visual field narrowing and spectacles that diminish the light have been useful in patients with retinal degenerations. The Macular Photocoagulation Study group in 1991 showed a treatment benefit for laser photocoagulation compared with no treatment only for a select subgroup of patients with AMD complicated by subfoveal choroidal neovascularisation (Penfold 2001). However, there was a significant postoperative reduction in visual acuity from laser injury.

1.6 Medical intervention

A minor subset of retinal degenerations can be helped with medical intervention. These include controlling the serum phytanic acid levels in Refsum disease by dietary adjustment (Stokke 1986) or controlling the serum ornithine levels in gyrate atrophy of the choroid and retina (Valle 1980) and treatment of A-β-lipoproteinaemia with fat-soluble vitamins (MacGilchrist 1988). Vitamin A was shown to slow the course of retinal degeneration by using Electroretinogram (ERG) evidence in RP while vitamin E increased the rate of retinal degeneration (Berson 1993). Antioxidants have been shown to be beneficial in AMD by Seddon et al. (Seddon 1994) whereas no association was found between the two by other investigators (Mares-Perlman 1996 and Smith 1999). Age-Related Eye Disease Study Research Group (AREDS) found that in the AMD trial groups, those at high risk of developing advanced AMD lowered their risk by about 25% when treated with the mega
dosages of the combination of antioxidants and zinc (AREDS 2001). D-cis-diltiazam has been shown to rescue photoreceptors and preserve visual function in retinal degeneration (rd) mice (Frasson 1999).

Many growth factors, once injected intra-vitreally, have been shown to slow photoreceptor loss in several retinal degeneration rodent models (La Vail 1992a &1998, Faktorovich 1990& 1992), suggesting the possible use of these agents for therapeutic intervention. However, growth factors are non-specific mitogens and may lead to vasoproliferation (Hicks 1991) and vitreous scarring (Fredj-Reygrobellet 1989). Since long-term treatment is likely to be needed by these patients, growth factors are required to be delivered to the eye in a sustained and controlled manner such as in slow-release polymers. Both the delivery mechanisms and potential side effects of the growth factors prevent their usage in treatment of hereditary retinal degeneration.

Since the final common pathway of photoreceptor degeneration in both AMD and RP is apoptosis, some researchers suggest that over-expression of genes such as bcl-1 (Sharma 1999) and IL-1β-converting enzyme (Janicke 1996, Takahashi 1996) in the retina may lead to prevention of apoptosis.

1.7 Genetic treatment

Genetic treatment aims at either introducing a healthy gene in to the genome or changing the expression of the mutated genes. Transferring genes into the retina requires the ability to introduce them into the post-mitotic non-dividing photoreceptors and RPE cells. However, if the transfer of genes occurs early enough, some promising results ensue. A study performed where the rhodopsin gene was transferred into the genome of one-day-old embryos, where the cells are still actively dividing, led to the rescue of photoreceptor degeneration (Lem 1992, Travis 1992). Injecting non-replicating adenoviral vectors carrying bFGF into the post-mitotic photoreceptors and RPE cells in vivo by subretinal injection have also led to the PR rescue (Akimoto 1999). It appears that despite the immense technical difficulties, it is possible to introduce a new gene into the genome but the expression of the gene product may not be sufficient to stop the disease as in the case of the autosomal dominant RP where a normal copy of the gene is already present (Sharma 1999). Furthermore, most studies performed using the adenoviral vector to transfer growth hormone (GH) genes found loss of label and photoreceptors after a short duration possibly due to
instability of the transgenes. Although most studies did not detect viruses in the blood or
other organs, Dudus et al. found persistent transgene product after its introduction into the
eye (Dudus 1999). Accumulation of the product of an abnormal gene may in itself lead to
pathogenesis. Recently, the use of anti-sense RNA sequences, positive sense corruption and
gene targeting techniques have enabled investigators to reduce the expression of abnormal
genes (Sharma 1999). The problems of introduction of a gene, prolongation of its expression
and prevention of viral disease need to be solved before this modality of treatment becomes a
viable option.

1.8 Microelectrode prosthesis

Implanted microphotodiodes are used as an alternative to photoreceptors in order to
carry visual signal to the cells of the inner retina. They have been placed subretinally to
stimulate bipolar cells (Peyman 1998) or vitreally to stimulate ganglion cells (Eckmiller
1997). Although these implants appear to remain stable (Peyman 1998), their efficiency at
transmitting signals to the progressively deteriorating retina is questionable. The ability of the
CNS to interpret the signals received from such prostheses is unknown and the best one can
hope for is perhaps a crude navigational vision (Lund 2001).

1.9 Cell transplantation

1.9.1 History of retinal transplantation

The first known ocular transplantation occurred in 1887 where a rabbit eye was
transplanted into a human eye for cosmetic reasons without success, as one can imagine. The
first retinal transplantation was performed by Tasley in 1946 who studied retinal
differentiation of embryonic ocular tissue transplanted into brains of young rats (Tansley
1946). Royo and Quey placed foetal rat retina into the anterior chamber of the maternal
parent (Royo 1959). Del Cerro et al. have been responsible for the first serious wave of
interest in retinal transplantation in mid 1980s when they transplanted full thickness retina
into the anterior chamber of a mouse and demonstrated good survival in both allo and
xenografts (del Cerro 1985). Turner and Blair later transplanted neonatal rat retina into the
subretinal space using a trans-scleral approach (Turner 1986). The laboratory of Lund and
colleagues implanted foetal rat retinas to the brain of neonatal rats. These grafts produced
axons originating from ganglion cells and connecting to the superior colliculus in the brain stem. Upon illumination, these grafts led to constriction of the pupil of the host’s eye (Coffey 1990, Simons 1985, Radel 1995)

1.9.2 Approaches to cell transplantation

The approach to cell transplantation includes transplantation to prevent photoreceptor loss and transplantation of photoreceptors to replace those lost. Cells have been transplanted into the eyes of RCS rats, a model of retinal degeneration, rd mice (model of retinitis pigmentosa) and other rhodopsin mutant as well as normal rodents. Larger animals such as rabbits (Seiler 1990, Ghosh 1998), cats (Huang 1998) and monkeys (Gouras 1984, Gouras 1985, Abe 2000) have also been used both as donors and as recipients of transplanted grafts.

In order to prevent photoreceptor death, to date fresh (Li 1991), cultured (Lopez 1987, Sheedlo 1993)), cryo-preserved (Durlu 1997) or immortalised retinal pigment epithelium (Keegan 2000) as well as iris pigment epithelium (Rezai 1997a, Schraermeyer 1999), stem cells (Takahashi 1998, Chacko 2000, Young 2000, Van Hoffelen 2003) and Schwann cells (Lawrence 2000) have been transplanted into the subretinal space. To replace the photoreceptors already lost or at least to place cells that may support diseased photoreceptors, two approaches have been made: a) to graft freshly isolated embryonic, foetal or adult retinas, or b) neural progenitor cells.

1.9.3 Transplantation of RPE

RPE transplantation has been reported to rescue the degenerating photoreceptors as well as preventing neovascularisation in the RCS rats (Li 1988, Lopez 1989, Seaton 1992). Transplanted RPE cells demonstrate an increase in number of materials ingested into phagosomes leading to the conclusion that phagocytosis maybe important in the rescue effect of photoreceptors (Lopez 1989). Moreover, only the photoreceptors overlying the grafted RPE are rescued as evidenced by the fact that only the photoreceptors with transplanted RPE interface showed a normal rod outer segment renewal rate (Lavail 1992). The photoreceptors thus rescued contain a normal distribution of Na/K-ATPase and normal amount of opsin (Silverman 1989). Furthermore, Jiang et al. demonstrated that rats, which had received allogeneic RPE transplants showed “rescued corneal ERG function” as apposed to non-grafted retinal dystrophic RCS rats (Jiang 1994).
Once RPE is in place, there is electro-microscopical evidence that they send delicate processes that can extend over a long distance to reach the outer segment of photoreceptors (Gouras 1998). There is also multiple evidence for the presence of trophic factors, for example basic fibroblast growth factor (bFGF), which also leads to photoreceptor rescue (Faktorovich 1990). Most allografts in the larger animal models have been performed in rabbits, where a suspension of RPE cells from brown pigmented rabbits have been injected into the subretinal space of albino rabbits both via a posterior scleral approach and via an anterior pars plana vitrectomy approach. These RPE transplants have been shown to survive for prolonged periods of time and form junctional complexes between the transplanted cells and also between host and grafted RPE (Crafoord 1999, Gouras 1992). Although some investigators found that the transplanted cells can form a monolayer, most transplantation leads to formation of clumps of cells between the host RPE and outer segment of photoreceptors. It is also difficult to remove the host RPE and position the transplanted cells precisely over the denuded area. The debridement of host RPE by bleb detachment can lead to a loss of the apical processes of RPE (Sheng 1995, Berglin 1997) and therefore other methods such as the silicone brush needle (Hayashi 1999) or application of an Ca^{++}-chelating agent together with gentle brushing (Del Priore 1995) have been used to remove host RPE.

RPE transplants survive best and are more likely to attach to the Bruch’s membrane if they are transplanted as a monolayer. Foetal RPE from human and pigs have been successfully transplanted into the subretinal space as patch grafts (Sheng 1995, Ho 1996). Adult RPE easily dissociates making it difficult to be transplanted as a sheet. To overcome the problems associated with injection of cell suspension of RPE, several laboratories have attempted to organise the RPE into a monolayer using different support materials.

1.9.4 Materials used to transplant a sheet of RPE into the subretinal space

Porcine RPE grown on porcine and bovine corneal extra cellular matrix (ECM) or porcine anterior lens capsule (ALC) have successfully been transplanted into the subretinal space of pigs where the lens capsule was tolerated for at least 2 weeks (Nicolini 2000). Kiilgaard et al. also transplanted ALC into the subretinal space of allogeneic pigs and noted that the transplants were well tolerated without inflammation if the Bruch’s membrane was kept intact. Host RPE and glial cells covered the ALC after 11 days but if the Bruch’s membrane was damaged, the authors witnessed ingrowth of choroidal vessels and fibroblasts (Kiilgaard 2002). Thumann et al. cultured both bovine and porcine RPE on autologous
Descemet membrane and found that the cells formed a monolayer with cell-to-cell contact and some evidence of correct polarity (Thumann 1997).

Tezel and colleagues seeded human RPE onto different layers of Bruch’s membrane and noted that cultured RPE reattaches best to the basal lamina layer (BL), and displays higher proliferation rate and mitotic index when plated onto BL. The rate of proliferation and reattachment as well as the mitotic index decreases if deeper layers of the Bruch’s membrane are used. RPE plated onto the inner collagenous layer or elastin layer eventually died whereas those on BL repopulated within 2 weeks (Tezel 1999a). Ho et al. developed a technique for harvesting and transferring native the extracellular matrix to provide a substrate onto which RPE cells attach prior to transplantation into the subretinal space. They found that RPE attaches better and displays a lower apoptotic rate when grown on ECM as compared to bare culture plates (Ho 1996).

Other investigators have used artificial support to conserve RPE as a monolayer by growing RPE in vitro on collagen (Gouras 1994a) or biodegradable polymers (Giordano 1997, Lu 2001) or embedding the cells in gelatin (Ho 1996). RPE has also been cultured on “thermally responsive polymer porous substrates” (von Recum 1998) and shown to be able to form a confluent monolayer with tight junctions and polarised morphology. When the temperature of insert was reduced to 20°C, the cell cultures detached as an oriented polarised sheet. Collagen inserts are thick with poor degradability and permeability. Rezai et al. cultured sheets of human foetal RPE (HFRPE) on poly (DL-lactide-co-glycolide) polymer films and formed HFRPE spheroids. The spheroids were transplanted into the subretinal space of New Zealand albino rabbits and the polymer alone into the subretinal space of Dutch Belted pigmented rabbits. The bare polymer dissolved within 3 weeks without evidence of inflammation. The RPE spheroids, however, led to immune cell infiltration. The authors demonstrated that RPE can be grown on polymers and these can be successfully transplanted into the subretinal space. Although they observed immune cell infiltrations, this was not caused by the presence of polymer (Rezai 2000).

1.9.5 Transplantation of Neuronal Retina

In order to replace lost or damaged photoreceptors, some investigators have transplanted neural retina into the subretinal space: Embryonic retina (Aramant 1988 and 1994, Seiler 1990), dissociated retinal cells (del Cerro 1988 and 1989), photoreceptor sheets
(Silverman 1989, Schuschereba 1992) and retinal micro-aggregates (Gouras 2003) have been transplanted into the subretinal space. In general, embryonic retinal grafts fare better than postnatal retina (Lund 2001, Turner 1986, del Cerro 1988) and placement of a sheet of retina with correct orientation resulted in better integration of transplanted material with the host retina (Ghosh 2000, Sharma 2000).

Transplantation of cell suspensions or fragments of embryonic or neonatal photoreceptors led to cell survival and differentiation in the subretinal space of the host. These grafts, however, formed rosettes rather than the normal linear morphology with debatable host-graft connections. Gouras et al. transplanted micro-aggregates of neural retina from transgenic mice containing LacZ-labelled photoreceptors into the subretinal space of normal and rd mice. The authors discovered that if the photoreceptors were transplanted into the subretinal space with correct orientation, they survived with normal outer segments and synaptic terminals for nine months post transplantation. In rd mice, the transplanted photoreceptors re-formed the host photoreceptors layer while in normal mice, they replaced the host photoreceptors layer, which subsequently degenerated. Areas of possible contact between donor and host neurons were found (Gouras 1994b). Kwan and co-workers also found integration between the grafted retinal cell and the surviving host retina in rd mice as evidenced by multiple photoreceptor synapses and the ability of the transplanted animals to perform light-dark preference test (Kwan 1999).

Full thickness and vibratome-cut partial thickness embryonic and adult retinas from rabbits were transplanted into the subretinal space of allogeneic rabbit by Ghosh and Ehinger. Embryonic rabbit retinas survived for at least ten months without immunosuppression and exhibited straight laminated morphology with correct polarity and all the layers of the retina present (Ghosh 1999b). They noted that the grafts fused with the host retina at the level of the inner plexiform layer and exhibited sprouting of both host and graft amacrine cells (Ghosh 1999b). Larsson and colleagues transplanted foetal neural retina of rats into allogeneic and syngeneic subretinal space of other rats and found that unlike the syngeneic transplants, marked up-regulation of class I MHC and numerous class II positive cells were seen in the allogeneic transplants and in the host retina at 5 weeks. Allogeneic transplants were, however, not rejected despite their MHC up-regulation (Larsson 1999).
1.9.6 Xenogeneic transplantation

Freshly isolated adult porcine RPE was placed into the subretinal space of RCS rats. Compared to sham injection or no injection, there was a short-lived but significant rescue of photoreceptors as evidenced by the maximum layers of surviving photoreceptors. The same authors noted that although xenogeneic RPE transplants were vigorously rejected subcutaneously, those placed in the anterior chamber and subretinal space did not exhibit strong immune rejection but a slower “functional deterioration” (Grisanti 2002).

Rauer and Ghosh placed complete immature rat retina into the subretinal space of adult rabbits without immunosuppression. After 35 days, surviving grafts were found in five out of nine eyes, three of which displayed laminated normal retinal appearance and the other two had developed rosettes. The survival was associated with the state of host RPE and the levels of MHC present. While in the surviving grafts RPE was present as a continuous layer, there were multiple defects in the host RPE of the failed grafts. Surviving grafts had minimal MHC up-regulation while the choroid, subretinal space and neural retina of the host containing failed grafts expressed an increase in MHC class I and II labelling (Rauer 2001).

Human foetal RPE xenografts were placed into the subretinal space of monkeys by Berglin et al. The authors noted that the grafts survived for at least six months in the monkey subretinal space, formed a basal lamina and made contact with the outer segments of photoreceptors of the host. Eventually, 60% of foveal and 30% of peripheral transplants were rejected (Berglin 1997). RPE derived from cadaveric eyes of a 10 year old and a 49 year old donor was placed in the subretinal space of RCS rats that received daily cyclosporin injections. Both RPEs promoted photoreceptor survival.

It is interesting and a testament to the value of the immune privilege in the subretinal space that even xenografts survive for prolonged period of time.

1.9.7 Measurement of the ability of transplants to function

The success of most of the transplantations into animals with retinal degeneration is judged by their survival and integration with the host. In order to determine the ability of transplants to restore function to the grafted eyes, several approaches have been developed to measure visual functions in animal models. In RCS rats, the ERG response is lost by 60 days of age and progressive visual field loss to focal stimuli is seen at around 40 days and is complete by 200 days. By 300 days of age, the RCS rats even lose responses to whole field
illumination (Lund 1997). There is a reduction in the animal’s photopic response leading to the avoidance of brightly illuminated areas. This response is progressively reduced in rats (Lund 1997). RCS rats also lose orienting response mediated through the superior colliculus.

Transplanted rats have been evaluated using several of these methods. ERG measurement has already been discussed (Jiang 1992, Yamamoto 1993). Whitely et al. noted an improvement in both latency and amplitude of the pupillary light reflex following transplantation (Whiteley 1996). Sauve et al. noted improvement in the visual field to focal stimuli after RPE transplantation (Sauve 2002). Coffey and co-workers transplanted a human RPE cell line into RCS rats and using head tracking to moving stripes and pattern discrimination together with single-unit cortical physiology, demonstrated that transplantation can preserve cortically mediated vision (Coffey 2002).

1.9.8 Retinal transplantation in humans

There are a few laboratories that have experimented with transplantation into the human. They have aimed to study: a) Feasibility and safety of different methods of cell transplantations; b) survival of transplanted materials with or without immunosuppression; and c) visual outcome of patients following transplantation. They have placed both RPE and retinal tissues, in a sheet or dissociated, foetal or cadaveric into the subretinal space of patients.

Peyman and associates transplanted allogeneic RPE-Bruch’s membrane explants or translocated autologous retinal pigment epithelium after sub-macular scar excision in two patients with AMD. Visual acuity improved in one patient, but the graft became encapsulated without visual acuity improvement in the second patient (Peyman 1991).

Algvere and associates removed the subretinal membrane and performed the transplantation of a patch of foetal RPE into the subretinal space of five adult patients. All transplants survived for three months and grew to cover the epithelial defect left as the result of membrane removal. Originally, some visual function was noted over the transplant but cystoid macular oedema (CMO) ensued and the grafts became encapsulated by white fibrous tissue within several months after surgery (Algvere 1994). The same group later transplanted suspensions of foetal human RPE into five patients with exudative AMD and four patients with dry AMD after membranectomy and without immunosuppression. In eyes with exudative AMD, cystoid macular oedema developed with reduction of visual acuity. In
contrast, grafts placed in the eyes of patients with non-exudative AMD remained the same shape and size and showed no evidence of rejection (Algvere 1997). Algvere et al. transplanted allogeneic foetal human RPE into the subretinal space of 14 patients with AMD and 2 patients with RPE tears (Grierson 1994). They discovered that only small patch transplants placed over the fovea in eyes with non-exudative AMD survived for up to 2 years. All others with concomitant breaks in the BRB were rejected (Algvere 1999).

Del Cerro transplanted a foetal neural retinal sheet as well as a retinal microaggregate suspension into the subretinal space of a 94-year-old with neovascular AMD. Three years later, the histological section of the eye revealed survival of transplanted cells with layered configuration and without any evidence of inflammation. No surviving RPE was noted over the grafted area and much of the posterior pole. The patient did not show any visual improvement (del Cerro 2000). A gelatin-embedded sheet of RPE from a cadaveric donor was transplanted into the subretinal space of an 85-year-old woman after she underwent subfoveal membranectomy for bilateral exudative AMD. Post-operatively, the patient was immunosuppressed with prednisone, cyclosporin and azathioprine. The presence of large pigmented cells was observed and identified as macrophages in the subretinal space of the recipient associated with loss of host photoreceptor outer segment and RPE (del Priore 2001).

Kaplan and colleagues transplanted sheets of human photoreceptor cells from two cadaveric eyes into the subretinal space of two non-immunosuppressed patients with advanced retinitis pigmentosa. Although there was no difference in the visual acuity of the patients, no evidence of rejection of allogeneic material was noted and no surgical complications such as cystoid macular oedema, uveitis and macular pucker was observed (Kaplan 1997). Intact sheets of human foetal retina (15-17 weeks) were placed into the subretinal space of two patients with advanced RP. At around six months post-operatively, both patients reported new visual sensation over the visual field corresponding to the transplant, which continued for 8-12 months. Although the patients were not immunosuppressed, no evidence of rejection was noted (Radtke 1999). The same group transplanted combined foetal RPE and neuronal retina into the subretinal space of five patients with advanced RP and noted the growth of two of the transplants over six months without evidence of rejection (Radtke 2002). Das et al. transplanted cell suspensions of human foetal neuroretinal cells into the subretinal space of 14 patients with advanced RP without immunosuppression. Apart from one patient who developed retinal detachment, the
grafts survived for 12-40 months without any evidence of inflammation, infection or graft rejection (Das 1999).

1.9.9 Transplantation of neural progenitor cells

Stem cells are defined as self-renewing multipotent cells that are able to regenerate. Many mammalian tissues including the retina and brain have recently been shown to contain stem cells, which can be grown in culture, differentiated, and grafted into hosts (Ahmad 1999, Tropepe 2000). Stem cells acquire the phenotype of cells of the tissues into which they are placed. For example, neural stem cells that were transplanted intravenously into irradiated hosts differentiated into bone marrow cells (Bjornson 1999). More importantly, stem cells are able the respond to injury, since they were shown to replace lost oligodendrocytes in mutant shiverer mice (Yandava 1999). Transplantation of embryonic stem cells appears to delay photoreceptor cell degeneration in RCS rats (Schaermeyer 2001, Takahashi M 1998; Young 2000, Wojciechowski 2002).

Young et al. reported the first use of neural progenitor cells taken from adult rat hippocampus progenitor cells into the eyes of rats with a genetic retinal degeneration. After survival times up to 16 weeks, the retinas of 1-, 4-, and 10-week-old recipients exhibited widespread incorporation of green fluorescent protein-expressing (GFP-positive) donor cells into the host retina. The 18-week-old recipients showed similar patterns, but with fewer cells (Young 2000).

Lu et al. isolated EGF-responsive neurospheres from the brains of green fluorescent protein transgenic mice and transplanted them into the eyes of mature rd mice, a model of retinitis pigmentosa. EGF-responsive neurospheres were also implanted into the retina of mice with a photoreceptor-specific defect and their behaviour was compared with that of cells taken from early postnatal retina. While grafts of freshly isolated postnatal day 8 retinas expressed many markers characteristic of mature retina (e.g. rhodopsin, protein kinase C), very few of the grafted cells migrated into host retina. EGF-responsive neurospheres, conversely, readily migrated into and integrated with the remaining host retina, but showed a very limited ability to differentiate into mature retinal neurons (Lu 2002). Embryonic stem cells from the inner cell mass of the mouse blastocyst were allowed to differentiate to neural precursor cells in vitro and were then transplanted into the subretinal space of 20-day-old RCS rats. In the eyes transplanted with embryonic cells up to 8 rows of photoreceptor cell
nuclei were observed, whereas in non-treated control eyes the outer nuclear layer had degenerated completely (Lu 2002).

Both neural and retinal progenitor cells tend to integrate better with the host retina and since they can renew themselves repeatedly, they appear to be promising cells for future retinal transplantation. In many cases described above, transplants survived for prolonged periods of time in both allogeneic and xenogeneic settings. This is partially contributable to the fact that the subretinal space is an immune privileged site (Jiang 1993, 1994 and 1995, Wenkel 1998). However, long-term survival of many transplants is uncertain.

1.9.10 The immunological fate of grafts in retinal transplantation

Only a few laboratories have investigated the immunological fate of grafts placed in the subretinal space. Jiang et al. showed that neonatal RPE allografts from C57BL/6 placed in the anterior chamber and the subretinal space of BALB/c mice survived for prolonged period of time (Jiang 1994a). Once these recipients of the neonatal RPE and retinal grafts into the anterior chamber were challenged again by the donor antigen, the RPE grafts were rejected with intense inflammation. The retinal grafts, however, deteriorated without much evidence of inflammation suggesting that there is an unconventional immune response in play.

Zhang and Bok injected freshly harvested RPE cells from neonatal rats into the subretinal space of the allogeneic RCS rats. Two weeks later they challenged the recipients with donor splenocytes. The extent of photoreceptor rescue was measured by counting the maximum layer of surviving photoreceptor nuclei histologically. They discovered that a chronic rejection occurred more readily in the immunologically challenged rats as evidenced by a decrease in the number of surviving photoreceptor cells. Grafts with disparity at both MHC class I and II had a worse outcome as compared with the grafts with disparity only at MHC II. They further discovered that the transplanted RPE cells, which were negative for class II at the time of transplantation, became positive after placement in the subretinal space (Zhang 1998). Larsson and co-workers also made the observation that allogeneic foetal neural transplant from rats led to up-regulation of class I and II in the subretinal space of the host (Larsson 1999). Up-regulation of IL-1α, IL-1β, IL-2, IL-6, IFN-γ and TNF-α has been noted after human to rat neuronal retina, RPE and IPE transplants (Abe 1999). As described above, grafts placed in the subretinal space of patients with exudative AMD fared worse with more
complications than those placed in the subretinal space of patients with non-exudative form (Algvere 1997).

There are issues of the foetal versus adult retina and single cell suspension versus an intact sheet of retina. Blood vessels and with them microglia migrate into the retina only postnatally in rats and from 16 weeks of gestation in human. Foetal retinal grafts have therefore far fewer microglial cells than postnatal grafts (Ashwell 1989). Transplantation of dissociated RPE cells have been shown to lead to chronic rejection and an increase in class I and II MHC expression (Algvere 1999, Zhang 1998). Intact sheet of RPE may have the additional advantage of acting as an immune privileged tissue (Wenkel 2000).

The above experiments point to the fact that the rejection of retinal and RPE grafts placed in the subretinal space is immunologically based. This is further supported by the study performed by Aramant et al. that showed transplanted sheets of human retina and RPE survive indefinitely in Nude rats and develop normally (Aramant 2002) and by the fact that cyclosporin treatment promotes survival of human foetal neural retina transplanted into the subretinal space of light damaged rats (DiLoreto 1996). Local immunosuppression by intravitreal injection of cyclosporin was also found to prolong survival of xenografts in the subretinal space.

Transplantation of autologous iris pigment epithelium (IPE) has been used to eliminate the rejection factor in transplantation. IPE has been shown to phagocytose photoreceptor outer segments (Schraermeyer 1997) and to delay photoreceptor degeneration in RCS rats (Rezai 1997b). Crafoord and co-workers demonstrated the ability of fresh autologous IPE to survive in the subretinal space and integrate with the host RPE (Crafoord 2001). Autologous IPE harvested from a peripheral iridectomy was injected as a single cell suspension into subretinal space of 12 patients after removal of subfoveal CNV. Post operatively up to 6 months the patients’ visual acuity remained stable but without improvement (Lappas 2000).

It is clear that there are many obstacles in the road to producing a “perfect” transplant: One with easy access to donor material, transplanted with surgical success, without leading to immune rejection, enjoying good integration with the host, at an early enough time in the life of the host to prevent total loss of photoreceptors and above all to produce functioning “seeing” eyes.
With the advent of stem cell transplantation and availability of immortalised RPE cell lines and with development of biodegradable polymers that may make it possible to transplant an intact sheet of RPE to the subretinal space, we maybe on our way to solving the problem of access to donor materials. Surgical expertise is growing rapidly in the area of transplantation and there seems to be more evidence, particularly in case of stem cells, of host-graft interaction. The formidable barrier of immune rejection, however, still remains. Most allografts placed in the subretinal space enjoy prolonged survival since the subretinal space is an immune privilege site. However, they ultimately fail either through lack of integration or through a chronic immune rejection.

In this thesis, emphasis has been put on understanding the mechanisms that govern the immune privilege of the subretinal space. The main “actors” in this play are the cells and molecules of immune system as well as parenchymal eye derived cells that face the subretinal space. Before understanding how the interactions between various players affect the immune privilege of subretinal space and ultimately the success of transplantation, some of these players are introduced here. The cells and molecules of immune system are discussed in greater detail in subsequent chapters.

1.10 Innate and adaptive immunity

The innate immune system comprising of a large number of molecules and cells is the first line of defence against the offending pathogens. Unlike the adaptive immune system, the innate defence mechanisms pre-exist in individuals and the response rate is rapid. Neutrophils, macrophages, dendritic cells, natural killer cells (NK), and natural killer T cells (NKT) are the major cellular players all of which are able to recognise and respond immediately to pathogens.

Adaptive immune response is defined as an immune reaction that develops after the infection has begun and the host organism adapts to the microbe. There are two types of adaptive immunity: cell-mediated and humoural immunity. Adaptive or acquired immunity has several cardinal features that distinguish it from the innate, preformed immunity:

a) Specificity: Immune responses are specific for each antigen or even for a different part of a complex protein ensuring that specific microbes elicit distinct response.
b) Diversity: Lymphocytes have a large number of antigenic specificity called “lymphocyte repertoire” enabling the immune system to respond to a large variety of pathogens.

c) Memory: The adaptive immune system produces clones of lymphocytes specific to the encountered antigen named memory cells. Each subsequent time that the antigen is encountered, the immune system mounts a quicker response.

d) Specialisation: Depending on the type of pathogen or antigen encountered, the immune system mounts responses optimal to defence against that organism.

e) Self-limitation: All immune responses eventually diminish to allow the system to return to homeostasis and become prepared to respond to new pathogenic invasion.

f) Non-reactivity to self: In normal individuals the immune system is “tolerant” to self-antigens preventing injury to the self. The breakdown of this safeguard leads to autoimmune disease.

Lymphocytes are the mediators of the adaptive immunity and are the second most common (1500-2700/μl of blood) and the smallest of the leucocytes in human. There are two subsets of lymphocytes known as T and B cells. B cells are responsible for humoral responses of the immune system through secretion of antibodies. T cells, named after they were found to be thymus dependent, are responsible for cell-mediated immunity as well as performing a modulating role on B cells.

Detailed description of all the cells and molecules of the immune system that are relevant and important in immune reactions to the transplanted materials in the subretinal space is not possible here. Where relevant in the subsequent chapters, these cells and molecules will be discussed in greater details.

1.10.1 Afferent and efferent limbs of the cellular immune response

The afferent limb of cellular immunity defines the process of antigen-specific T cell activation to an offending antigen. Foreign antigens are transported on the surface of APCs into a secondary lymphoid organ, where they are presented to T cells in the context of MHC on surface of the APCs. T cells possess receptors on their cell surface that recognise antigens and are the basis for specific immune responses. T cell receptor (TCR) is a cell surface receptor that requires processing of the antigen and its binding to the MHC molecule prior to recognition. TCR then interacts with the antigen and additional co stimulatory signals
produced either by the APCs or the microenvironment where this encounter is taking place. The antigen recognition leads to a cascade of changes in biochemical status of T cells resulting in T cell proliferation, maturation and differentiation, and initiation of the immune response.

The efferent limb of the immune system is the process by which activated T cells travel and gain entry to the site of inflammation. Once there, activated T cells perform their function through delayed hypersensitivity (DH), cytotoxicity and antibody production. Activated T cells exert their influence by producing cytokines, and by up-regulating integrin expression and affinity whilst decreasing selectin expression. Hence instead of T cells homing to the secondary lymph nodes (L-selectin mediated function in naïve T cells), they interact with vascular endothelium at the inflammatory site to which they become “tethered”. Following the initial tethering, the inflammatory cells must bind firmly to the vascular endothelium which they do so via interactions of two families of inducible adhesion molecules: a) β integrins and VLA-4; and b) intercellular adhesion molecule-1 (ICAM-1) and Vascular adhesion molecule-1 (VCAM-1), members of the Ig superfamily. Pro-inflammatory cytokines produced by leukocytes such as TNF-α and TGF-β up-regulate ICAM-1 and VCAM-1 expression on vascular endothelium. It is believed that interaction of leukocytes with vascular endothelium at inflammatory sites leads to phenotypic and functional changes in the leukocytes enhancing their migration through the extracellular matrix of tissues.

Once in the inflamed tissue, T cells are reactivated either by professional APC (B cells, macrophages and LC) that constitutively express class II MHC or by resident tissue cells. The professional APC also provide a co-stimulatory signal required for T cell activation. The resident tissue cells include epithelial and endothelial cells that do not constitutively express class II but can be induced to do so by IFN-γ stimulation, which often occurs at the site of inflammation.

**Regulatory T cells**

Once the immune response has achieved its end of destroying or deactivating the offending antigen, further colonal expansion and effector cell activity must be limited in order to achieve homeostasis. Regulatory or suppressor T cells perform this function. CD4+ CD25+ T cells are the naturally occurring population of suppressor T cells found in the thymus and lymph nodes. They are able to suppress the proliferative responses of both CD4+ and CD8+
CD25⁺ T cells by a contact-dependent mechanism. CD25⁺ T cells mediate their effect by inhibiting the induction of IL-2 mRNA in the responder CD25⁺ T cells leading to induction of cell cycle arrest. Activated CD4⁺ CD25⁺ T cells inhibit both T cells proliferation and IFN-γ production by CD3 activated T cells in the absence of APCs.

Apart from the naturally occurring CD4⁺ CD25⁺ T cells population, multiple populations of both CD4⁺ and CD8⁺ suppressor cells have been produced after certain in vivo or in vitro treatment protocols. These regulatory cells share some of the characteristics of the natural regulators. Low dose administration of antigen via the oral route has resulted in formation of population of regulatory T cells that secrete suppressor cytokines. The environment in which T cells are primed is critical in determining their ultimate fate: low expression of co-stimulatory molecules on APCs or the presence of suppressor cytokines, such as TGF-β or IL-10 leads to formation of regulatory instead of effector T cells (Barrat 2002). Drugs, such as vitamin D3 and Dexomethasone act by inducing populations of IL-10 producing T cells by inhibiting transcription of nuclear factor of activated T cells (NF-AT), activator protein 1, and NFκB (Paul 2003a, Barrat 2002).

Regulatory T cells have been shown to have a role in suppressing organ specific autoimmune diseases (Gleeson 1996, Powrie 1994). They have a role in control of allograft rejection (Waldman 2001) and participate in prevention of tumour immunity (North 1984).

1.11 Transplantation immunology

The earliest known tissue transplantation was performed by Hindu surgeons who repaired an amputated nose by using the patient’s own forehead skin flap in 700 BC (Paul 2003b). Subsequently many surgeons tried, often unsuccessfully, to transplant various organs both from syngeneic donors and allogeneic donors (Paul 2003b). In the 1900s while studying tumour transplantation, Little made several seemingly confusing observation, which led to the formation of the genetic “laws of transplantation” (Paul 2003b). Sir Peter Medawar and his colleagues were the first to establish the relationship of clinical transplantation to the field of immunology and define the immunological rules that govern transplantation in 1940s and 1950s.

Others have added to the wealth of information that we posses about transplantation today. The fundamental rules, however, remain the same: Foreign tissue grafts placed in
recipient display transplantation antigens. Recipient T and B-lymphocytes express surface receptors that recognise these antigens. Once the graft is in place, antigenic materials from the graft via blood/lymphatic drainage reach secondary lymphoid organs. There the antigens are presented to T and B cells via antigen presenting cells (APC). The lymphocytes then become activated, clonally expand and differentiate into T helper cells; cytotoxic T cell and T cells mediating delayed hypersensitivity. The effector cells and molecules then return to the site of original graft via blood circulation. At the graft site, they leave the circulation, recognise the antigen again and initiate a destructive inflammatory response. The process of inflammation not only is able to destroy the grafted material but also destroys some of the recipient’s tissues.

During the course of his experiments, Medawar discovered that tolerance to non-self can be acquired leading to long-term acceptance of transplanted graft (transplantation tolerance). There are multiple mechanisms that may explain transplantation tolerance depending on whether the graft is vascularised or not; on the type of lymphoid cells encountered by the graft and whether MHC class I or II or non-MHC factors pose barriers to acceptance of the grafts. Transplantation tolerance can be divided into: central and peripheral tolerance. Central tolerance occurs in the thymus and is the mechanism by which the immune system destroys T cells that are strongly auto-reactive by a process called clonal deletion. Central tolerance is not perfect as T cells that are only mildly reactive to self, escape notice of the immune system. Peripheral tolerance is the process by which different tissues of the body modulate their interaction with the immune system according to the needs and vulnerability of the tissues. Some sites or tissues take this relationship further to develop the concept of immune privilege.

1.12 Immune privilege

The diversity of pathogens causing disease is enormous and each pathogen exploits hosts’ vulnerabilities to its advantage. Once the innate immune system is triggered, an intense inflammation invariably ensues in order to eliminate the pathogens. Adaptive immune effectors such as T cells mediating delayed hypersensitivity also employ innate immune cells such as macrophages and activate them to release the content of their granules which are both lethal to micro organisms and to neighbouring cells. The aim of immune privilege is to
suppress bystander damage of innate immunity and control the “expression” of adaptive immunity.

The concept of regional immunity is based on the fact that pathogens are specific for the tissues and organs they attack and each site has a different vulnerability to the bystander damage (Streilein 1996). Tissues, therefore, influence and direct the type of immune reactions best suited to their offending pathogens and their vulnerabilities. Tissues exert this control by: the type of antigen presenting cells present which transport the antigen to the secondary lymphoid organs and the type of T cells which then home to these tissue sites; the afferent pathway of antigen delivery to the secondary lymphoid organs and the efferent pathway by which the effector cells reach the tissue site; and the microenvironment produced by factors released from the parenchymal cells of the tissue site.

The eye exerts a unique influence on the immune system defined by immune privilege. Medawar first coined the term immune privilege in 1948 (Medawar 1948). He used the term to identify experimental situations where allografts placed in specific sites in the body were found to be resistant to immune rejection whereas they would be readily rejected if placed subcutaneously. These sites include the anterior chamber, vitreous cavity and subretinal space within the eye, and the brain, the gonads, adrenal cortex, and the maternal-foetal interface during pregnancy. Factors that allow immune privilege to exist are: anatomical and physical features, immunosuppressive properties of ocular cells, soluble immunosuppressive factors in ocular fluids, expression of transplantation antigens and the special way that the adaptive immune system acts in the eye (Streilein 1999b).

All the immune privilege compartments of the eye lie behind blood-tissue barriers. Therefore, in normal eyes, immune effector cells and molecules (antibodies, complement components, clotting factors) have limited access into these compartments. These barriers in effect interrupt the efferent limb of the immune response. None of these compartments possess appreciable lymphatic drainage and in the case of the anterior chamber, the antigenic material present in aqueous humour drains directly into the venous circulation through the trabecular meshwork. These antigens end up in the spleen instead of regional lymph nodes and therefore primarily lead to expression of humeral immunity rather than cell associated immunity. The response of the spleen to soluble antigens is to usually produce non-complement fixing antibodies. It is also interesting that antigens entering the spleen
predominantly produce regulatory T cells that suppress induction of immunity as well as other T cells that inhibit the expression of both cellular and humoral immunity.

Antigens injected into the anterior chamber elicit a deviant immune response termed Anterior Chamber Associated Immune Deviation (ACAID). ACAID is characterised by synthesis of non-complement fixing antibodies, generation of cytotoxic T lymphocyte precursors, and absence of delayed hypersensitivity (DH). The impaired DH coincides with survival of immunogenic tumor allografts placed into the AC (Streilein 1999). Even though DH and complement fixing antibodies are inhibited, the body still mounts a systemic immune response to ocular antigens by production of non-complement fixing antibodies and primed cytotoxic T cells. Bill and associates demonstrated that if the uveoscleral pathway of aqueous humour drainage is opened through treatment with topical PGF$_2$α, subsequent injection of OVA into the anterior chamber does not elicit ACAID. Since the OVA antigen from the uveoscleral pathway flows into the lymphatic system, this result emphasises the importance of predominant escape route of antigens within the eye to be through the circulation into the spleen. ACAID-like phenomena exist in the vitreous cavity and subretinal space and contribute to immune privilege (Streilein 1980, Streilein 1987, Niederkorn 1990, Niederkorn 1981, Streilein 1993, Streilein 1999b). ACAID is also produced against non-soluble antigens. P815 mastocytoma cells injected into the anterior chamber and subretinal space of histo-incompatible BALB/c mice elicited the phenomenon of ACAID (Wenkel 1999).

Antigen presenting cells comprised of dendritic cells (DC) and macrophages restricted to the eye, are found in all compartments of the eye including the central cornea. These APCs however are incapable of functioning in a conventional manner leading to T cell activation. The evidence for this comes from several studies. Williamson et al. made a single cell suspension of iris and ciliary body (I/CB), which included DCs but failed to activate allo-reactive T cells (Williamson 1989). A relatively pure population of dendritic cells from I/CB produced by Bradley and colleagues also failed to present antigen to T cells (Streilein 1991) and even treatment with sub inflammatory dose of IFN-γ did not lead to T cell activation (Streilein 1992). Caspi et al. demonstrated the same phenomena with Müller cells in the posterior segment (Caspi 1987). Although the evidence points to the fact that these DCs lack conventional APC capacities, they nevertheless function as specialised ocular APC. Wilbanks et al. demonstrated that F480+ macrophages pick up intraocular antigens and via the trabecular meshwork enter the circulation and then the spleen (Wilbanks 1992). These cells
are not able to activate DH inducing T cells either in vivo or in vitro but induce a population of splenic T cells that suppress DH (Niederkorn 1990, Streilein 1987, Tompsett 1990).

Epithelial cells of the iris, ciliary body, RPE and corneal endothelium secrete immunosuppressive factors, which inhibit T cell activation (Yoshida 2000). Corneal endothelium and epithelium, keratocytes and RPE constitutively express CD95 ligand that upon cross linking with the CD95 receptor on T cells lead to the apoptosis of T cells. AqH that bathes the anterior chamber contains a variety of factors leading to immunosuppression. These include α-MSH (Taylor 1992), IL-1RA (Mo 1998), TGF-β (Cousins 1991), MIF (Apte 1997), VIP (Taylor 1994), thrombospondin (Sheibani 2000), CGRP (Wahlestedt 1986), CD95L, cortisol (Knisely 1994), somatostatin (Taylor 2003), soluble CD55 (Sohn 2000), CD59 (Sohn 2000) and CD46 (Sohn 2000). While TGF-β2 and VIP mediate anti-proliferative properties of aqueous humour, α-MSH inhibits IFN-γ production by activated T cells (Streilein 1999a).

Any inflammation and its resolution into scar formation can lead to opacification of visual axis and hence loss of clarity to the path of light and vision. It has thus, been hypothesised that the presence of ACAID and the ability of the eye to direct the immune system away from inflammation has an evolutionary advantage in preservation of sight.

### 1.13 Experimental autoimmune diseases and immune deviation

Since it appears that immune privilege exists in order to prevent inflammation, it is logical to assume that immune privilege is lost during inflammatory eye diseases. In a series of studies performed in the laboratory of Streilein, immune privilege in the context of inflammation was investigated. As described above, immune privilege is measured by the presence of ACAID, prolonged survival of allogeneic tissue grafts, and by the presence of immunosuppressive microenvironment implied by inhibition of T cell activation in vitro and the integrity of blood ocular barriers. Uveitis was produced either by systemic (foot pad) injection of bacterial lipopolysaccharide (LPS) into susceptible C3H/HeN mice to produce systemic endotoxin-induced uveitis (s-EIU) or injected intravitreally into normal BALB/c mice at a smaller dose to produce local endotoxin-induced uveitis (l-EIU). L-EIU produces a more intense anterior uveitis, has an earlier onset and a longer course than s-EIU. The same investigators also studied more chronic models of uveitis by intravitreal injection of mycobacterial adjuvant into BALB/c mice leading to production of mycobacterial adjuvant-
induced uveitis (MAIU) and by systemic immunisation of the susceptible B10.A mice with IRBP, pertussis and CFA to produce a chronic pan uveitis termed experimental autoimmune uveitis (EAU) (Streilein 2002b).

The breach in blood ocular barrier (BOB) was measured by the amount of protein and cellular infiltration in the anterior chamber of the eye. In all forms of inflammation there is a breakdown of blood ocular barrier as evidenced by a rise in protein levels and presence of inflammatory cells. The time line for the initial loss of BOB and eventual reforming correlated well with the chronicity of the disease. In s-EIU, the aqueous humour transiently lost its ability to inhibit T cell activation due to loss of activity of α-melanocyte stimulating hormone (α-MSH) and vasoactive intestinal peptide (VIP). The high level of IL-6 found during the peak of the disease was blamed for the transient loss of immunosuppressive microenvironment (Ohta 2000b). Although very high levels of IL-6 accompanied by intense protein and cell infiltration were also found in I-EIU, the aqueous humour taken at the peak disease was able to inhibit T cell activation. Moreover injection of OVA into the anterior chamber (AC) of these mice at the peak of inflammation led to induction of ACAID. Similarly P815 tumour cells injected into the AC at the peak of inflammation grew progressively and induced P815-specific ACAID (Mo 2001). It therefore appears that unlike systemically injected LPS, locally injected endotoxin fails to eliminate immune privilege despite a more severe inflammation. It maybe that local delivery of an offending antigen modulates the immune response to that antigen. Hara et al. found that, unlike systemic immunisation of B10.A which led to development of EAU, if the immunising agents were injected into the AC of these animals, the immune system was altered in such a way as to abolish the ability of these animals to mount a DH response (Hara 1992). Moreover, these animals did not exhibit appreciable autoimmune inflammation. Ohta and co-workers also discovered that unlike the AqH of the normal eye, the inflamed eyes contained active TGF-β and it was the presence of this cytokine that led to restoration of immunosuppressive microenvironment (Ohta 2000a).

MAIU led to the transient loss of ACAID associated with a rise in IL-12. P815 tumours, however, grew progressively in these eyes and the AqH retained the ability to suppress T cell activation (Streilein 2002b). Unlike EAU, which is mediated through Th1 effector cells, in all other forms of uveitis mentioned the prevailing cell population were neutrophils and mononuclear cells with different preponderance depending on the type of
uveitis. In EAU, the immune privilege of AC was lost transiently as evidenced by their inability to inhibit T cell activation at day 11 and failure to support ACAID at peak disease.

It appears that ocular immune privilege is mainly able to persist through an inflammatory response and that it uses various strategies to overcome and limit the destructive inflammatory processes. The rules that govern immune privilege at a specific site differ from those existing at other site. Put differently every immune privilege site chooses specific strategies to confer immune privilege. Attention must therefore be paid to specific strategies used by the subretinal space.

1.14 The subretinal space as an immune privileged site

The subretinal space is a potential space that exists between the intervagination of the outer segments of the photoreceptors with the apical surface of the RPE (Figure 1.1). This space is a remnant of embryonic optic vesicle and in the normal adult eye is very small. However, no tissue junctions are able to form across it and it becomes open and filled with subretinal fluid in rhegmatogenous retinal detachment. In this condition fluid from the vitreous cavity reaches subretinal space through a retinal hole causing the separation between the outer segment of the photoreceptors and RPE. The subretinal space is usually filled with interphotoreceptor matrix, a hydrophilic matrix made up of proteoglycans and large glycoproteins (Mieziewska 1996).

1.14.1 Blood retinal barriers

The neuroretina is separated from the circulation by the BRB (Ashton 1965). The BRB is comprised of two distinct components: the inner BRB is made by the retinal vascular endothelia and the outer BRB by the tight junctions between RPE cells. The zonula adherents that exist between Müller cells and photoreceptors at the base of the outer segment, producing the outer limiting membrane, contribute to the barrier function by limiting the movement of large molecules (Marmor 1999). Together, these cellular barriers control the passage of molecules and cells into and from the retinal tissues and are essential in maintaining homeostasis of the ocular environment. In effect these barriers produce a microenvironment, isolated from the circulation, where immune reactions are attuned. In most diseases of the BRB, both the RPE and the retinal capillaries are affected regardless of the original site of BRB breach (Marmor 1999). In fact there is evidence that breakdown of outer BRB occurs
very early in the course of EAU with the earliest cell infiltrate being CD4+ T cells present at the level of photoreceptors (Liversidge 1998a).

Immune privilege of the SRS is dependent on the integrity of the BRB. This is evidenced by abolishment of privilege when tight junctions between the RPE cells are interrupted after i.v. injection of sodium iodate (Wenkel 1998). The integrity of the BRB is undermined in the cell transplantation setting in many different situations. The surgical procedure that places the transplanted material into the subretinal space leads to the breakdown of inner or outer BRB or both. Moreover the surgical intervention is associated with inflammation and a rise in pro-inflammatory mediators. It has been shown that injection of IFN-γ into the vitreous of rats and rabbits leads to a leukocytic influx into the retina (Lee 1990, Hjelmeland 1992) through a breach in the BRB (Martiney 1990). When Zech et al. treated a cultured monolayer of rat RPE with LPS, IFN-γ and TNF-α, they found that both the transepithelial resistance and the level of tight junction-associated protein zonula occludens-1 (ZO-1) were lowered after treatment as compared to the untreated controls (Zech 1998). Mechanical stretching of RPE, which may occur at the time of surgery, has been shown to increase production of VEGF in culture (Seko 1999) and sustained intravitreal release of VEGF in vitreous has been shown to causes widespread breakdown of the BRB (Ozaki 1997).

Since the transplanted materials are placed in retinal degeneration models, the fact that diseased retinas of RCS rats and rd mice display leakage and disruption in the BRB (Villegas-Perez 1998, Wang 2000) is of significance. Chang et al. demonstrated that RPE from RCS rats are capable of producing normal tight junctions if they were treated with conditioned media of normal retinas. When RPE from RCS rats were co-cultured with retinas also from RCS rats, the RPE were unable to form tight junctions. Trophic factors released from normal retina, it seems, are essential in maintenance of the normal BRB (Chang 1997). Breakdown of the BRB is also noted in patients with rod-cone dystrophy as demonstrated by increased fluorescence across the BRB (Fishman 1986).
Figure 1.1: Diagrammatic representation of the retina and the subretinal space

Choroid
Bruch's membrane
Pigment epithelium
Subretinal space
Outer segment of photoreceptors
Müller cell
Outer plexiform layer
Outer nuclear layer
Inner plexiform layer
Ganglion cells
Optic nerve fibers
1.14.2 Evidence that immune privilege exists in the subretinal space

The laboratories of Jiang and Streilein have been the main groups investigating the phenomena of immune privilege in the subretinal space. They placed allogeneic neonatal retinal grafts from C57BL/6 mice into the subretinal space, vitreous cavity and subconjunctival space of BALB/c mice. They noted that the neonatal retinal grafts placed in the subconjunctival space were destroyed by post transplantation day 12 with the recipients displaying intense donor specific delayed hypersensitivity. By contrast, grafts placed in the vitreous cavity and subretinal space survived for prolonged periods of time and formed rosettes without any evidence of inflammation; the recipients did not display donor-specific DH and the regulatory T cells in the spleen of the recipients were able to adaptively transfer this phenomenon (Jiang 1995). Similar results were noted for cultured neonatal RPE cells from C57BL/6 recipients being placed into the SRS and AC of BALB/c mice (Jiang 1993, Grisanti 1997). By day 35 post implantation into the subretinal space however, the grafts reduced in size and lost their organisation and the recipients displayed donor-specific DH (Jiang 1995).

Since in all the above studies, either retinal tissues or retinal pigment epithelium were placed into the subretinal space and since both these tissues are known to be immune privileged, it was difficult to determine the reason for their prolonged survival in the subretinal space. To address this ambiguity, Wenkel et al. placed P815 mastocytoma (DBA/2 background) tumour cells into the subretinal space of BALB/c mice and demonstrated that compared to their placement sub-conjunctivally, P815 tumours grew progressively in the anterior chamber and the subretinal space until day 14, further confirming the presence of immune privilege in the subretinal space (Wenkel 1998). Subsequently, the grafts placed in the anterior chamber continued to grow, leading to the eventual demise of the animals but the grafts placed in the subretinal space regressed after 14 days leading to phthisis or tumour elimination and with preserved ocular anatomy without the demise of the animal (Wenkel 1999). Wenkel also demonstrated that there is immune deviation to both soluble (OVA) and cellular (P815) antigens in the subretinal space and in the case of BALB/c recipient of P815 tumours, but tumour elimination did not abrogate ACAID. In contrast to the anterior chamber, the antibody production to E1-deleted virus (Bennett 1996) and P815 mastocytoma tumour (Kaplan 1999b) is markedly diminished in the subretinal space. These studies further demonstrate that although immune privilege exists in the subretinal space, it is not absolute.
and the mechanisms for this privilege differ from the immune privilege in the anterior chamber.

As previously described the presence of immunosuppressive microenvironment is essential in the maintaining immune privilege. The end feet of Müller cells abut the subretinal space and as they have been shown to inhibit T cell proliferation (Caspi 1989), Müller cells may participate in providing the microenvironment of the subretinal space. Glial cells present in the retina also contribute to the deviant way in which the immune system is controlled in the subretinal space. They will be discussed in subsequent chapters. The immunosuppressive microenvironment of the subretinal space is primarily produced by the RPE monolayer that forms the outer limit of the subretinal space. RPE is implicated in a variety of retinal diseases and the health and integrity of this monolayer have been found to be essential in the maintenance of immune privilege of the subretinal space. In the subsequent segment the structure and properties of this monolayer of cells is discussed.

1.15 RPE

1.15.1 Historical perspective

During ancient times, the retina was considered to be an accessory structure carrying nourishment to the important vitreous and lens. Averroes in the 10th century and Kepler in the 17th century developed the idea that the retina is important in photoreception. After the development of techniques in histology, retinal pigment epithelium (RPE) was discovered in the second half of the 18th century (Marmor 1998). RPE is directly or indirectly involved in several diseases of the retina: Sorsby’s fundus dystrophy and two forms of autosomal recessive RP are due to a defect in the RPE layer itself (Della 1996, Gu 1997). A defective gene is expressed strongly in the RPE of patients with Usher’s syndrome (Hasson 1995). Others, such as AMD and Stargard’s, affect the RPE at early stages (Allikmets 1997). Generalised genetic defects such as choroideraemial lead to blindness due to their early effect on RPE (Gouras 1998).

1.15.2 Embryology

During embryogenesis, the optic vesicle grows out of the ventrolateral forebrain neuroepithelium (Morse 1984) and invaginates to form the optic cup. The inner and outer
layers of the optic cup give rise to the neural retina and RPE respectively and the margins of
the two layers become the ciliary epithelium.

1.15.3 Position, histology and polarity

RPE is a monolayer of polygonal cells interposed between the choroid and the neural
retina. In humans, the RPE cells are smaller in the macular region and enlarge towards the
periphery. Since the density of photoreceptors also reduces from posterior pole to the
periphery, there is an almost constant ratio of photoreceptors to RPE (45:1) (Zhao 1997).

In vivo, like all epithelial and endothelial cells, mature RPE shows morphological and
functional polarity along their apical-basal axis (Zhao 1997). The basal plasma membrane is
attached to Bruch's membrane and is markedly infolded. The apical surface has numerous
microvilli that partially enclose the photoreceptor outer segments. The apical cytoplasm
contains microfilaments and microtubules and the largest accumulation of melanin in the
RPE. The apical surface forms unusual junctional complexes with the lateral wall, which
consist of a mixture of tight, adherence and gap junctions. In contrast to most epithelia, the
Na/K-ATPase localises to the apical membrane, whereas the chloride-bicarbonate exchange
transporter is located on the basal membrane (Marmor 1998a). RPE preferentially produces
and secretes various factors across its basal or apical surfaces. Cultured bovine RPE cells
secrete retinal binding protein (RBP) and transthyretin (TTR) predominantly across the apical
membrane (Jaworowski 1995), whereas VEGF is secreted mainly via the baso-lateral surface
where its receptor KDR is located in the endothelium of choriocapillaries (CC), suggesting a
role for VEGF in a paracrine relationship between RPE and CC. RPE is shown to have a
positive survival effect on CC but preserves the avacularity of the photoreceptor layer and
therefore, the polarised secretion of VEGF seems to be important to preserve this dual
function of RPE. Both IL-8 and IL-6 are secreted preferentially from the basal surface and
although TGF-β has a polarised secretion pattern, the direction of secretion is variable
between different donors (Holtkamp 1999b).

1.15.4 Functions of RPE cells

Due to their neuroectodermal origin, RPE cells have dual nature: their epithelial status
is related to their polarity and transport functions; and their glial-like activity manifests in
phagocytosis of the outer segments (Grierson 1994).
RPE cells are involved in many essential activities:

a) They form tight junctions with one another and thus serve as the outer blood-retinal barrier.

b) The RPE provides physical protection for photoreceptors by their interdigitating processes.

c) RPE cells phagocytose shed outer segment of rods and cones and synthesise numerous enzymes to digest these debris.

d) Melanin granules in RPE cells absorb light, thereby reducing scatter and leading to a sharper retinal image.

e) Melanin in the RPE is capable of binding to heavy metal ions, which may provide the retina with a protective mechanism against oxidative damage by sequestering these ions.

f) RPE cells synthesise and transport many substances including GAG and vitamin A metabolites to and from retinal cells and the choroidal circulation.

g) They actively transport water from apical to basal surface to control subretinal fluid accumulation.

1.15.5 Immune privilege status

Some tissues present in immune privilege sites display special characteristics that lead to their enhanced survival when placed into a non-immune privilege site. These are termed immune privileged tissues. Corneal grafts devoid of epithelium as well as testes grafts containing Sertoli cells placed beneath the kidney capsule were shown to survive for a prolonged period of time (Hori 1999, Bellgrau 1995). Wenkel and associates demonstrated that neonatal RPE grafts prepared from eyes of C57BL/6 mice and placed under the kidney capsule of allogeneic BALB/c strain, survived for a long period of time. In contrast, grafts taken from the conjunctiva of C57BL/6 mice, similarly placed, were rapidly rejected (Wenkel 2000). The ability of RPE grafts to survive for an extended period depended on their ability to express CD95L since RPE grafts from gld/gld mice, which do not express CD95L, were readily rejected. Despite the fact that these RPE cells sensitised the host, the immune privilege status of RPE was maintained.
1.15.6 Role of RPE in the immune response

RPE plays a complex and sometimes contradictory role in the regulation of immune system. It is strategically placed not only to act as an immunological barrier by providing the outer BRB but also by expressing cell surface molecules and by secreting soluble mediators that influence the immune system.

Production of immunomodulatory factors by RPE is known to play an important role in immune responses by stimulating the expression of the MHC molecules, adhesion molecules, FasL and many cytokines, both pro- and anti-inflammatory (Holtkamp 2001). RPE constitutively expresses mRNA and secretes anti-inflammatory cytokines such as TGF-β, IL-1RA and a variety of growth factors. However, RPE has to be induced to express mRNA and secrete protein for pro-inflammatory cytokines such as IL-1α, IL-6, TNF-α and GM-CSF.

TGF-β. Retinal pigment epithelium expresses mRNA for TGF-β1 and TGF-β2 and for TGF-β binding protein (Tanihara 1993). RPE expresses all of type I TGF-β receptors (TβR-I, ActR-I, ActR-IB, BMPR-IA, BMPR-IB, ALK-1) and some of the type II receptors (TβR-II, ActR-II and BMPR-II) (Obata 1999).

TGF-β produced by RPE has an autocrine and paracrine role in both RPE cell proliferation and proliferation of other cells. The TGF-β family mediates the suppression of RPE growth in vitro, which is dependent on cell density. TGF-β1, TGF-β2 and TGF-β3 were shown to inhibit the proliferation of the primary cultures of porcine RPE cells in a dose-dependent fashion but were unable to inhibit 10th passage cells (Lee 2001). Neutralising TGF-β by using anti-TGF-β antibody may lead to a more rapid growth of RPE in vivo (Tezel 1999b). Retinoic acid was found to inhibit RPE cell proliferation and melanin synthesis in chickens via induction of TGF-β (Kishi 2001). The inhibitory effect of TGF-β was blocked by mannose-6-phosphate (Lee 1999), which is also produced by RPE. Hyalocyte conditioned medium inhibits RPE cell proliferation partially through secretion of TGF-β (Lazarus 1996).

TGF-β plays a role in the regulation of plasminogen activation on the RPE cell surface. In RPE, TGF-β increased expression of plasminogen activator inhibitor-1 (PAI-1) in a dose dependent fashion but only at higher concentrations of the cytokine, urokinase
expression is enhanced. Siren et al. also noted that incubation of RPE cells with TGF-β led to induction of urokinase receptor expression and secretion as well as induction of membrane-bound urokinase-type plasminogen activator (uPA) activity and the release of active PAI-1 (Siren 1999).

**PGE2.** Prostaglandins (PG) are produced from arachidonic acid (AA) via the cyclooxygenase pathway. PGE2 was found to be constitutively expressed in cultured RPE cells and the expression was found to be increased if RPE was co-cultured with activated T cells (Liversidge 1993). PGE2 was found to have an inhibitory effect on antigen and mitogen-activated T cell proliferation in one study (Liversidge 1993), but found to play no role in T cell proliferation in another (Rezai 1999). The level of PGE2 was shown to be elevated in the AqH of eyes with inflamed uveal tissue induced by injection of S antigen. Cyclooxygenase inhibitors such as flurbiprofen or indomethacin reversed this increase and also limited vasodilatation and infiltration of PMNs (Jaramillo 1992). In microglia, PGE2 enhances the LPS induced IL-10 production but inhibits that of TNF-α (Aloisi 1999).

**MIF.** Macrophage migration inhibitory factor (MIF) is produced by activated T cells, macrophages (Calandra 1994) and pituitary cells (Bernhagen 1993). MIF has an important role in delayed hypersensitivity (DH) (Bernhagen 1996), counteracts glucocorticoid inhibition of T cell activation and IL-2 and IFN-γ production (Calandra 1995-1996). In the eye, mRNA and protein expression of MIF are reported in human corneal endothelial and epithelial cells and in iris/ciliary body epithelium (Matsuda 1996). Matsuda et al. also discovered that Müller cells, retinal astrocytes and RPE are the retinal sources for MIF production (Matsuda 1997). MIF is found in AqH and vitreous and its levels are known to rise in patients with uveitis and those with proliferative diabetic retinopathy (Kitaichi 1999, Taguchi 2001, Mitamura 2000). Kitaichi et al. also demonstrated that anti-MIF antibodies delay the induction and severity of EAU in LEW rats (Kitaichi 2000).

**CSF.** Granulocyte-macrophage-colony-stimulating factor (GM-CSF) is an important regulator of macrophages and neutrophils by enhancing their survival, maturation and activation (Holtkamp 2001). This is evidenced by the fact that transgenic mice expressing high levels of GM-CSF suffer from intraocular inflammation (Lang 1987). Cultured human RPE cells express mRNA and secrete protein for GM-CSF after stimulation with IL-1α, IL-1β, TNF-α and TGF-β (Planck 1993, Crane 1999). RPE cells also constitutively express mRNA and secrete macrophage colony-stimulating factor (M-CSF), which acts only on
macrophages. The production of M-CSF is enhanced after treatment with IL-1, TNF-α or conditioned medium from stimulated monocytes (Jaffe 1992a and 1995).

**Growth factors.** Several growth factors are produced by RPE cells including nerve growth factor (NGF) (Dicou 1994), brain-derived growth factor, neurotropin-3 (NT) (Ishida 1997), fibroblast growth factor (FGF) (Connolly 1992), platelet derived growth factor (PDGF) (Campochiaro 1989), and insulin-like growth factors (IGF) (Ocrant 1991, Waldbillig 1991 and 1992). Treatment of rat RPE with combination of IFN-γ and LPS increased production of NGF by the RPE cells and the levels of NGF produced correlated well with the susceptibility of the rat strain to uveitis (Dicou 1994). RPE produces several forms of FGF: Acidic FGF (aFGF), basic FGF (bFGF) (Sternfeld 1989), FGF-1 (Guillonneau 1997) and FGF-5 (Bost 1992). bFGF expression is induced after hypoxia (Khaliq 1995) and other stressful stimuli and the levels increase after treatment with IL-1 (Hackett 1997). Since RPE also expresses receptors for bFGF, it is suggested that this cytokine may act in an autocrine or paracrine manner (Sternfeld 1989).

**VEGF.** Vascular endothelial growth factor (VEGF) is produced by RPE cells both in vitro and in vivo. VEGF production by RPE is induced by high glucose concentration, progesterone stimulation, mechanical stress (Seko 1999) as well as stimulation with IGF-1. VEGF induces vascular permeability and angiogenesis and plays a part in inflammatory processes. Increased expression of VEGF is noted in RPE from eyes with AMD as compared to the control (Kliffen 1997). Since VEGF production by RPE cells increases as a result of mechanical stretching of RPE cells in culture, the increased production of VEGF may have a role in increased neovascularisation and changes in the BRB (Seko 1999).

**PEDF.** Pigment epithelium-derived factor (PEDF) is secreted by RPE in early embryogenesis and is present in the the extracellular matrix between the photoreceptors and RPE. PEDF is a 50KD protein first discovered in the conditioned media of cultured RPE as a factor that induced neuronal differentiation in cultured Y79 retinoblastoma cells (Tombran-Tink 1991). Subsequently PEDF was found to be present in photoreceptors, the interphotoreceptor matrix, the inner nuclear layer and the ganglion cell layer by in situ hybridisation and by immunohistochemistry using antibody to PEDF in choroid, corneal epithelium and ciliary body (Karakousis 2001). Ortego et al. demonstrated that ciliary body synthesises and secretes PEDF into the AqH (Ortego 1996).
PEDF is neurotropic for cerebellar granular cells and also protects them from glutamate toxicity and induction of apoptotic cell death (Taniwaki 1997, Araki 1998). PEDF activates metabolism of microglia and RAW macrophage cell line while at the same time inhibits their proliferation (Sugita 1997, Cohen 1999).

Both recombinant and purified PEDF have been found to be potent inhibitors of neovascularisation in the cornea where they inhibited endothelial cell migration more so than angiostatin, thrombospondin-1 and endostatin (Dawson 1999). PEDF also inhibited migration of endothelial cells induced by VEGF, IL-8, aFGF and PDGF (Dawson 1999). Moreover, in absence of exogenous inducers of angiogenesis, addition of neutralising anti PEDF antibody led to invasion of new vessels into the retina. High levels of PEDF are also found in the vitreous sufficient to inhibit endothelial cell migration even in the presence of large doses of VEGF (Dawson 1999). Treatment of vitreous fluid with antibody to PEDF revealed an endogenous tendency to produce new vessels. The amount of angiogenic inhibitory function of PEDF produced by retinal cells was correlated with oxygen concentration, producing most inhibition in normal and high oxygen concentrations (Dawson 1999). Decreased production of PEDF is noted in aging RPE and may play a role in the formation of subretinal neovascularisation in AMD (Hjelmeland 1999).

**IL-1.** The pro-inflammatory cytokine IL-1 is a potent multifunctional cell activator and acts as a "proximal" mediator in a variety of immune responses (di Giovani 1990). There are two forms of IL-1, a secretory IL-1β and the cell surface associated IL-1α. IL-1β is cleaved into an active form by the IL-1β converting enzyme (ICE). Human RPE expresses mRNA for both IL-1α and β, but only after stimulation with IL-1, TNF-α or LPS (Jaffe 1992b) and produces only IL-1α and not IL-1β at the protein level.

IL-1 can induce RPE cells to produce immunomodulatory molecules such as IL-6, IL-8, and RANTES. IL-1 receptor antagonist (IL-1ra) is a naturally occurring antagonist that binds the receptor with the same affinity as IL-1β and α, but does not trigger signal transduction. Unstimulated and phorbol 12-myristate 13-acetate- (PMA) exposed RPE expresses mRNA for both intracellular (icIL-1ra) and secreted (sIL-1ra) variant of IL-1ra. However, at the protein level RPE produces mainly the intracellular type (Holtkamp 1999).

**IL-6.** IL-6 is a cytokine produced by lymphocytes, monocytes and macrophages as well as fibroblasts, endothelial and epithelial cells (Le 1989, Hirano 1990). Bacterial and viral
products and other cytokines such as IL-1, TNF-α and GM-CSF induce IL-6 expression. Once produced, IL-6 induces differentiation in macrophages and T cells and activates both T and B cells. IL-6 is considered as one of the early markers of acute organ dysfunction after transplantation (Boros 1997). RPE can secrete IL-6 only after stimulation with IL-1 and, less potently, with TNF-α (Planck 1992). The combination of IFN-γ and LPS, but not LPS alone, leads to secretion of IL-6 by RPE in a dose and time dependent manner (Benson 1992, Elner 1992). The secretion of IL-6 is polarised to the basal surface of the RPE, which may play an important role in immune processes between the RPE and choriocapillaries (Holtkamp 1998).

Enzmann et al. demonstrated that human RPE cells treated in vitro with either TNF-α or IFN-γ or combination of the two up-regulated their production of RANTES, IL-8, MCP-1 and IL-6 (Enzmann 1999). They then placed RPE grafts from rabbits, either activated with IFN-γ or untreated, into the subretinal space of pigmented rabbits and measured IL-6 levels in the vitreous at various time intervals. Compared to the sham injection, both transplanted groups showed an elevation of IL-6 in the vitreous and the sera. They noted that the level of IL-6 detected was dependent on the status of the host and the phase of rejection (Enzmann 2000). Thus, it appears that IL-6 produced by RPE cells may play an important role in the immune reactions to transplanted material in the subretinal space.

TNF-α. Tumour necrosis factor α (TNF-α) is a pro-inflammatory cytokine, secreted by monocytes and macrophages and is essential in the initiation of immune response. The effects of TNF-α closely resemble those of IL-1 and may be mediated through IL-1. TNF-α mediates chemotaxis and cell adhesion and mainly regulates cell migration during inflammation. High TNF-α levels have been found in vitrectomy specimens from eyes with AIDS-associated retinitis (Hofman 1992) and in the aqueous samples of LPS-induced uveitis (de Vos 1992). Human RPE cells have been reported to express mRNA for TNF-α but not to produce the protein after treatment with IL-1β or PMA (Tanihara 1992). Another study demonstrates TNF-α production by rat RPE in a constitutive manner (de Kozak 1994).

1.15.7 Chemokines

IL-8. IL-8 is produced by monocytes/macrophages, endothelial cells and fibroblasts and has a strong chemotactic and activating property for neutrophils (Baggiolini 1989). IL-8 is expressed in human RPE after stimulation with IL-1β, TNF-α, LPS (Strieter 1992) or IL-7 (a cytokine produced by bone marrow cells, fibroblasts and keratinocytes) (Elner 1996). IL-7,
IL-4 and glycosylated albumin enhanced the IL-1β/ TNF-α effect on IL-8 secretion (Elner 1996, Bian 1996) while dexamethasone and cyclosporin A inhibited it (Kurtz 1997). Treatment of cultured RPE with conditioned medium from activated T cells or monocytes produced IL-8, which was inhibited if the activated T cells were pretreated with antibodies to IFN-γ, TNF-α or both (Elner 1997). The secretion of IL-8 is polarised to the basal/choroidal side (Holtkamp 1998), which may lead to attraction of neutrophils to the choriocapillaries but prevents their migration across the RPE protecting the neuroretina from damage.

GRO. Another chemokine, growth-related-oncogene (GRO), which is a potent chemoattractant for neutrophils, is secreted by RPE after treatment with IL-1β and TNF-α (Jaffe 1993, Shattuck 1994) and by conditioned media from cultured monocytes (Jaffe 1995).

MCP-1. Monocyte chemotactic protein 1 (MCP-1), a chemokine primarily chemotactic for lymphocytes and monocytes, is produced by monocytes, epithelial and endothelial cells (Baggiolini 1994). Unstimulated RPE cells express mRNA for MCP-1 (Elner 1991). This expression can be up regulated in response to TNF-α, LPS, IL-1, IL-7 and glycosylated serum albumin (Bian 2001, Elner 1996). The induction and inhibition pattern of MCP-1 expression is governed by the same factors as those in IL-8. MCP-1 secretion is polarised towards the basal surface of RPE and during inflammation this may limit damage to the neurosensory retina (Holtkamp 1999b).

RANTES. Low levels of regulated on activation, T-cells expressed and secreted (RANTES) are produced constitutively by unstimulated RPE cells and its levels are up regulated as a result of IL-1β, and TNF-α (Crane 1998). RANTES acts on monocytes, basophils, eosinophils and memory T cells but not neutrophils.

1.15.8 Cell surface expression

CD95/CD95L. Fas ligand (FasL, CD95L) is a type II transmembrane protein and member of the TNF superfamily that upon cross-linking with Fas expressing cells leads to apoptotic death of these cells (Nagata 1995b). Fas (CD95) is expressed on multiple cell types but FasL expression is restricted to activated T cells, NK cells and parenchymal cells of immune privilege sites such as the eye, testis, brain and placenta (Nagata 1995b). The interaction of Fas with FasL is essential in maintaining the homeostasis of the immune system. The constitutive expression of FasL within the ocular tissues is thought to contribute to the immune privilege of the eye mainly by causing apoptosis in Fas-positive inflammatory...
cells (Griffith 1995). Kezuka and Streilein also showed that Fas-FasL interactions were essential in induction and expression of ACAID (Kezuka 2000). FasL can be cleaved by some metalloproteinases and released in a soluble form (sFasL). Soluble FasL was reported to antagonise the actions of the membrane bound variant and is found in large amounts in the AqH where it acts to support immune privilege (Gregory 2002).

RPE was found to express FasL using immunohistochemistry in vivo (Jorgensen 1998). Reports of FasL expression by cultured RPE are contradictory. Jorgensen et al. demonstrated FasL expression by flow cytometry and immunohistochemistry in cultured RPE cells and further showed that RPE induces apoptosis in activated T cells by a Fas-FasL dependent mechanism (Jorgensen 1998). Kaestel and co-workers, however, failed to find FasL expression by flow cytometry, western blotting, RT-PCR and RNAse Protection assay (Kaestel 2001). Rezai et al. also showed that RPE leads to apoptosis of T cells through a Fas-FasL independent mechanism (Rezai 1999). Expression of FasL by RPE cells was found to be essential for survival of neonatal RPE patches in the non-immune privilege environment of the subcapsular space of kidneys (Wenkel 2000).

RPE cells express Fas and apoptotic cell death can be induced in proliferating cultures of RPE cells (Chang 2001). Apoptosis of RPE may play a role in the pathogenesis of AMD. Dunaife et al. studied post-mortem retinas from AMD patients and noted that their maculas had a statistically significant number of TUNEL positive stained cells in the RPE, the inner choroid, photoreceptors and the inner nuclear layer. Additionally, photoreceptors at the edge of AMD defect were strongly Fas-positive (Dunaief 2002).

FasL on RPE has also been shown to have a role in regulation of growth and spread of subretinal neovascularisation (Kaplan 1999). Fas-positive vascular cells were found to be surrounded by FasL-positive RPE cells at the edge of the AMD defect in the clinical specimens from AMD patients. Moreover, cytotoxic antibodies against Fas prevent the formation of vascular tubules by the human choroidal endothelial cells (Kaplan 1999). This, together with the significant increase in the incidence and severity of new vessel formation in both Fas-and FasL deficient animals, points to the role of Fas-FasL interactions in the pathogenesis of neovascularisation.

By expression of FasL, the RPE inhibits neovascularisation from the choroid, contributes to its own immune privilege status as well as the immune privilege of the
subretinal space. The expression of FasL may be altered as RPE cells age, which may be related to neovascularisation associated with AMD (Kaplan 1999).

**Adhesion molecule expression and leukocyte transmigration.** Intercellular Adhesion Molecule-1 (ICAM-1/CD54) mediates leukocyte adherence and is present as a cell surface marker. ICAM-1 expression on vascular endothelial cells regulates the binding and transmigration of leukocytes (Holtkamp 2001) and its presence on various other cells controls extravascular lymphocyte trafficking. A secretory form of ICAM-1 (sICAM-1) is found in several inflammatory diseases including uveitis (Arocker-Mettinger 1992). Freshly isolated or cultured human RPE expresses surface ICAM-1 and secretes sICAM-1 with both variants being up-regulated after treatment with IL-1, IFN-γ or TNF-α (Liversidge 1990, Limb 1997, Nagineni 1996). After cytokine stimulation, RPE cells also expressed VCAM-1 (Platts 1995).

Adhesion molecules, in addition to regulating leukocyte traffic, influence other processes such as cellular growth and differentiation, polarity and junction formation (Liversidge 1998). Their presence and activation is essential in immune surveillance and accumulation of leukocytes at the site of infection.

ICAM-1, present on the surface of RPE, was shown to be functional in binding of both neutrophils (Elner 1992c) and lymphocytes (Liversidge 1990, Elner 1992c) to the RPE monolayer. While ICAM-1 appears to be the only molecule involved in neutrophil adhesion, other adhesion molecules maybe involved in lymphocyte adherence to RPE. Mesri et al. demonstrated that naïve T cells bind to unstimulated RPE cells in an ICAM-1 independent manner and that IFN-γ treatment of RPE cells did not increase the adhesiveness of T cells (Mesri 1994). Since antibodies to ICAM-1 and its ligand LFA-1 (CD18) block adhesion of T cells to RPE cells, it can be deduced that ICAM-1 plays a crucial role in adhesiveness of activated lymphocytes (Mesri 1994). Some inflammatory eye diseases, for example sympathetic ophthalmia, leads to *de novo* expression of adhesion molecules and the homing receptor CD44 (Whitcup 1992).

Devine et al. demonstrated that untreated peripheral lymph node (PLN) cells of rats can migrate through RPE only if the monolayer is activated with IFN-γ. However, once PLN cells and T cells were activated, they were able to cross the unstimulated monolayer of RPE. The migration of PLN through unstimulated RPE was blocked with ICAM-1 and LFA-1 antibodies but if the RPE layer was stimulated, the transmigration of T cells was blocked in addition to the above with antibodies to VCAM or its ligand VLA-4 (Devine 1996a).
**Integrins.** Integrins are transmembrane glycoproteins of the superfamilies of cell adhesion molecules that modulate cell adhesion to cells and to the extracellular matrix. They take part in wound repair, platelet aggregation, leukocyte trafficking, tumour invasion, embryogenesis and immunological responses. Integrins are heterodimeric proteins consisting of α and β subunits bound together non-covalently. There are 15 α and 8 β subunits identified, which combine in various configurations to give rise to 21 integrins. Each combination of α and β chain has one or more possible ligands, the specificity of which is determined by the β chain. Several factors such as TGF-β, TNF-α and IL-1β can increase the expression of integrins leading to enhanced cellular adhesion to collagen, laminin and fibronectin. Integrins are widespread throughout the eye. RPE has been shown to contain β₁, β₂, α₂, α₃, α₄, α₅ and α₆ subunits (Chen 1997, Robbins 1994, Rizzolo 1994). The integrins play a role in accumulation of thickened basement membrane material in diabetic microangiopathy (Roth 1993) and β₂ integrins containing α₅ or α₆ have been identified in human proliferative vitreoretinopathy (PVR) membranes (Robbins 1994). β₂ integrins were found to be essential in the pathogenesis of uveitis as evidenced by their presence in iris biopsies of patients with anterior uveitis (Elner 1996) and on the lymphocytes taken from patients with various forms of posterior uveitis (Elner 1996). The integrin α₅β₅ has been shown to reside on the apical surface of the RPE and has been implicated in the phagocytosis of shed photoreceptor outer segments. (Chen 1997).

**CD36 and other scavenger receptors.** CD36 is a platelet integral membrane glycoprotein, which participates in multiple biological processes as a “multiligand scavenger receptor” (Silverstein 2000). The best known ligand for CD36 is thrombospondin-1, but long chain fatty acids, anionic phospholipids, retinal photoreceptor outer segments and apoptotic cells, amongst others, are also found to be internalised through CD36 receptor (Yesner 1996, Ryeom 1996b, Ibrahimi 1996 Tait 1999, Ren 1995). CD36 expression in the body is broad and includes microvascular endothelia, platelets, monocyte/macrophages and dendritic cells (Yesner 1996, Tandon 1995, Montes 1996, Savill 1992).

CD36 expression was found in both human and rat RPE by immunohistochemistry, RT-PCR and western blot analysis (Ryeom 1996). Cross-linking of anionic phospholipids on photoreceptor outer segment with the CD36 receptors as well as α₅β₅ integrin receptors on RPE cells leads to recognition and internalisation of the outer segments by RPE (Ryeom 1996a). CD36 participates in rod outer segment phagocytosis by RPE cells and hence, in the
visual cycle. RPE cells of the RCS rat, in which the RPE fails to phagocytose outer segments of photoreceptors, express CD36 mRNA only transiently during postnatal development. The CD36 protein, however, is not expressed emphasising the important role of CD36 (Sparrow 1997). In addition, RPE has receptors for mannose 6-phosphate, a scavenging receptor, amongst others, which stresses the role that RPE plays as the first line of defence at the BRB in the absence of macrophages (McLaughlin 1987).

RPE cells were found in one study to constitutively express other antigens usually associated with blood derived monocyte/macrophage cells. All isoforms of CD45, the macrophage markers CD45RA, CD45R and CD45RO were found to be expressed by cultured RPE cells (Limb 1997). CD68 usually produced by monocytes and macrophages was also found in 10% of human RPE in vivo (Liversidge 1991) with an increase in expression after culturing. CD68 is associated with lysosomes and has a role in the digestion of phagocytosed material. Constitutive expression of these markers may aid RPE in its macrophage like function.

**CD40/CD40L (CD154), CD80 and CD86.** T cell activation usually involves 2 signals. One provided by engagement of T cell receptor (TCR) on APCs to CD3 on T cells and the other involves the interaction of co-stimulatory molecules B7.1 (CD80) and B7.2 (CD86) on the APCs with CD28 on T cells. Moreover, ligation of CD40, expressed by professional APCs (dendritic cells, B cells and macrophages), to CD40L, present on the surface of activated T cells, leads to up-regulation of co-stimulatory molecules on APC and high IL-12 secretion. This in turn induces increased antigen presentation and co-stimulation by APCs.

CD40 was found to be expressed by IFN-γ-activated RPE but not by resting human RPE. Neither CD80 nor CD86 were found to be present (Willermain 2000; Rezai 1997d). Ligation of CD40 was shown to increase IL-6 and IL-8 production but not induction of co-stimulatory molecules or production of IL-12 (Willermain 2000). These findings imply that although RPE cells are capable of acting as antigen presenting cells, in absence of co-stimulation, they may present the antigen in a deviant manner leading to suppression of the immune system.

**CD59 and CD48.** RPE cells strongly express CD59, an inhibitor of the cytolytic membrane attack complex (MAC), which may act to protect RPE from complement-mediated damage (Liversidge 1998a). In addition, CD59 is a potential ligand for CD2 activation of T
cells. Liversidge et al. demonstrated that CD59 from RPE can induce syngeneic T cell proliferation through a CD2 mediated pathway without the presence of exogenous antigen or mitogen (Liversidge 1996). RPE also expresses low levels of CD48, which takes part in the bi-directional activation pathway between RPE and T cells: CD59 on RPE leads to T cell activation and T cell ligation of CD48 on RPE cells leads to production of IL-1β and IL-6 required by T cells (Liversidge 1996).

**Other cell surface molecules.** The mRNAs for TNFRp55, TNFRp75, IFN-γRα, IFN-γRβ and TGF-βRII are found to be constitutively expressed *in vitro* in RPE cells. IL-10R mRNA was detected in neither unstimulated nor stimulated RPE cells (Hollborn 2001).

**Complement.** Complement receptors for C3b (CD35) and C3bi (CD11b/CD18) as well as the Fc portion of IgG have been found on fresh RPE cells (Elner 1981, Devine 1996a) reflecting the complex role that RPE plays in regulation of the immune system. C5aR has been found on the surface of the ARPE-19 cell line in a similar quantity to that found on human PMN (Fukuoka 2001). Upon C5a stimulation of these receptors, mRNA expression of IL-8 was shown to increase in a dose- and time-dependent manner. Since IL-8 is secreted from the basal surface of RPE towards the choroid, it maybe suggested that C5a has a role in protecting the choroidal and retinal tissue during inflammation (Fukuoka 2001). The same authors later showed that primary human RPE cell line (RPE43) as well as ARPE-19 expressed IL-1β, IL-6, MCP-1 and GM-CSF mRNAs as well as IL-8 mRNA after stimulation with C5a (Fukuoka 2003).

**1.15.9 RPE as a target of cytokine**

Cytokines in general act on RPE cells to induce production of more cytokines, production of nitrous oxide, expression of the MHC and adhesion molecules, and to aid transmigration of leukocytes across RPE. Cytokine production by RPE as a result of the action of other cytokines was noted previously.

**Nitrous Oxide.** Nitrous oxide (NO) is a gas with various biological functions such as cytotoxicity, vascular relaxation and signal transduction. Different isoforms of nitric oxide synthetase (NOS) exist with different tissue distribution and regulation of expression but all produce NO from L-arginine. cNOS is the constitutively produced isoform and participates in neurotransmission and vascular relaxation. The inducible form (iNOS) is produced by macrophages and neutrophils after LPS or cytokine stimulation. RPE cells can be induced to
produce NO after treatment with TNF-α or IFN-γ and LPS. In mice and humans, NO production is inhibited by TGF-β but in bovine RPE, TGF-β₁ increases the level of nitrite produced by RPE after stimulation with IFN-γ and LPS (Faure 1999). RPE cells from rat cocultured with activated lymphocytes produce high amounts of NO (Liversidge 1994), which may play a role in maintenance of immune privilege of the subretinal space by its cytotoxic effect on lymphocytes. Both TGF-β and TNF-α have a role in regulation of NO production by RPE cells as evidenced by the fact that NO production was increased in TGF-β knock out mice and reduced in TNF-α knock out mice (Liversidge 1994).

**MHC expression and antigen presentation** Various cytokines influence the MHC expression on RPE cells. RPE cell express class I MHC but their expression is not maximal and increases in response to IFN-γ (Benson 1992b). Freshly isolated RPE cells from adult murine RPE were found to be immunogenic at minor histocompatibility loci when they were tested with T cell clones in the ^51^Cr release assay (Kaplan 1999c). Cultured RPE cells can be induced to express class II MHC in response to IFN-γ or supernatant of culture medium from activated lymphocytes. *In vivo*, MHC class II expression has been noted on RPE cells of patients with uveitis (Chan 1986) and retinitis pigmentosa (Detrick 1985). TGF-β₁ and TGF-β₂ (Gabrielian 1994) and IL-10, on the other hand, inhibit the expression of IFN-γ induced class II expression on RPE cells. Since expression of class II on the surface of grafted RPE cells can lead to graft rejection, Enzmann *et al.* by using magnetic beads demonstrated that positive cells could be selectively removed increasing the chances of transplantation success (Enzmann 1998).

RPE cells activated by IFN-γ express class II and have been shown to act as antigen presenting cells. IFN-γ treated RPE was shown to increase T cell proliferation and IL-2 production by retinal antigen-specific T cells (Percopo 1990). This ability decreases if IFN-γ-stimulated RPE was additionally treated with TGF-β or IL-10 (Enzmann 2002). Human foetal RPE was unable to provide T cell activation in an anti-CD3 dependent manner but could induce an allo-response from T cells if APCs were also available, demonstrating that they are not very effective at antigen presentation due to lack of co-stimulation (Farrokh-Siar 1999). IFN-γ-treated primary cultures of human RPE cells that expressed class II MHC were able to bind staphylococcal enterotoxin E (SEE) and presented SEE to T cells. This was measured both by proliferation of peripheral blood T cells and by synthesis of IL-2 from the Jurkat T
cell line (Osusky 1997). Rezai et al. corroborated this observation and showed that human foetal RPE is able to provide both antigen presentation and co-stimulation for superantigen-mediated T cell activation (Rezai 1997d).

The ability of RPE to present antigen implicates them both in induction of acquired immunity and as a potential initiator of autoimmunity. During retinal injury, some photoreceptor antigens such as retinal S antigen, inter-photoreceptor binding protein and rhodopsin, amongst others, may be released. These antigens, when injected with adjuvant into susceptible animals, have been shown to produce EAU. The induced inflammation requires antigen processing by APC and presentation to CD4 positive T cells. Since RPE has the ability to present antigen and acquires the specific photoreceptor antigens through phagocytosis, it has been argued that in conditions where class II expression is induced in RPE cells, such as during inflammation, RPE may act as antigen presenter for auto-reactive T cells (Liversidge 1998c).

1.15.10 Interactions with cellular immunity

RPE cells are only able to present antigen to T cells when they are treated with pro-inflammatory cytokines and their class II MHC is up regulated. If cultured RPE cells are not treated with pro-inflammatory cytokines, however, they have been shown to profoundly inhibit T cell proliferation even in the presence of APCs (Liversidge 1993 and 1994). Jorgensen et al. reported that cultured foetal human RPE cells (HFRPE) incubated with anti-CD3 activated T cell population led to apoptosis of T cells in a Fas-FasL dependent manner (Jorgensen 1998). In the same study, they also noted that HFRPE induced significant apoptosis in the Jurkat cell line that do not express TCR molecules. Antibodies to class II MHC, which block recognition of alloantigens, had no effect on HFRPE-mediated induction of apoptosis. Moreover, the authors did not witness any apoptosis if the T cells were incubated with the supernatant of cultured HFRPE indicating that cell-to-cell contact is essential in the apoptotic function of RPE. Kaestel et al. also noted a cell contact-dependent inhibition of T cell activation, which correlated with a decreased expression of IL-2R-α and β chains (Kaestel 2002).

Rezai and colleagues subsequently corroborated the ability of HFRPE cells to suppress T cell activation by apoptosis. However, since they could not identify FasL either by flow cytometry or by RT-PCR on the cultured HFRPE cells, they concluded that Fas-FasL
interaction did not play any part in the RPE dependent T cell apoptosis (Rezai 1999). Additionally, anti-FasL antibodies did not block the inhibitory effect of RPE on T cells. RPE incubated with anti-CD3 activated T cells from lpr mice, which do not express Fas, still suppressed T cell activation. Since the cultured HFRPE were able to induce T cell apoptosis across the transwell membrane, the authors concluded that cell-to-cell contact is not required. Their search for the soluble factor by HFRPE cells excluded TGF-β, TNF-α or IL-10 since they were not able to find these by ELISA and their addition to the T cell culture did not lead to T cell apoptosis. Farrokh-Siar et al. further demonstrated that HFRPE cells inhibited the proliferation of Jurkat cells by apoptosis and that the passaging and treatment with IFN-γ increased their inhibitory effect. To prove that inhibition of T cell proliferation was induced through apoptosis, they co-cultured Jurkat cells that over-expressed Bcl-xI with HFRPE and noticed a reduction in RPE-mediated apoptosis (Farrokh-Siar 1999).

1.15.11 Cultured versus fresh RPE

Most studies performed on RPE and on the factors produced by it as well as many other structural and functional studies use cultured RPE cells. There is a wealth of evidence in the literature that is directed towards understanding the way RPE cells behave in culture and how this may differ from their behaviour while in vivo. The results of studies performed with cultured RPE maybe difficult to interpret and the physiological characteristics of RPE cells deduced may bear little resemblance to the in vivo situation.

For transplanted RPE to function as the protector of the blood retinal barrier, as the ingester of photoreceptor outer segments and as a provider of many factors necessary for the well being of the retina, RPE cells should remain polarised. Unlike other epithelia, confluent monolayers of RPE in culture are variable in shape and size and lack an ordered epithelial configuration (Zhao 1997). Epithelial cells are identified by compartmentalised cytoplasm and the asymmetrical presence of molecules on their surface to give them a polarised morphology. Organelles are positioned along the apico-basal axis and some plasma membranes are polarised to the apical, basal or lateral surface (Burke 1998a). This arrangement is sustained by cell-cell and cell-substrate attachment. This cell-to-cell contact controls the cytoarchitectural and polarity aspects of the cells and is mediated by the cadherin family, which together with circumferential bundles of actin produce zonular adherence junctions (Yonemura 1995).
In culture, single cell suspensions of RPE proliferate and form a monolayer. Several laboratories have found multiple morphologies of RPE cells, from epitheloid to fusiform, coexisting in the same culture. There is a suggestion that although RPE cells in vivo appear to be homogeneous, they are in fact heterogeneous populations that vary in their ability to form an epithelial layer in vitro. Epitheloid cultures of RPE have been shown to have apical microvilli and apical intracellular junctions, which points to some morphological polarity. RPE cells are found not to express E-cadherin in culture (Gundersen 1993) but they do so in situ (Burke 2000). Therefore, the cell-to-cell contact in cultured RPE does not appear to be normal. Frohlich et al. also noted marked variability between RPE cells on their secretion and activities of various enzymes such as aminopeptidases, alkaline phosphatase and cathepsin B (Fröhlich 2001).

In vivo, unlike other epithelia, the Na/K-ATPase on RPE is situated on the apical surface. Apart from the RPE cell line APR-19, which has apical abundance of Na/K-ATPase, other RPE cultures were found to have little or no enrichment of the sodium pump in their apical surface (Bok 1992, Nabi 1993, Hu 1994, Hernandez 1995). Membrane bound carbonic anhydrase, on the other hand, was restricted to the apical surface as is the case in situ (Wolfensberger 1994). Since E-cadherin is essential in polarising the sodium pump, its absence in cultured RPE cells may be the reason for lack of the pump polarity (Burke 1998b).

The culture conditions such as the provided substrate, presence or absence of other tissue interactions and soluble factors present in the culture medium, can change the molecular polarity of RPE. Addition of retinoic acid (Campochiaro 1991) and bFGF (Camporchiaro 1993) to cultures of RPE cells encourages the epitheloid morphology while EGF, NGF, PDGF and monocyte conditioned media lead to formation of a more spindle-shaped morphology (Martiney 1991). RPE cells grown on plastic fail to produce melanin and do not express cellular retinaldehyde binding protein (CRALBP). The phenotype of RPE cultured on basement membranes is more epitheloid in shape (Camporchiaro 1993, Song 1990) as compared with RPE cultured with collagen or vitreous humour (Docherty 1987, Heth 1987, Kirchhof 1988).

The length of the culture also affects the phenotype and function of RPE cells. Grisanti et al. noted that porcine RPE cells grown on plastic transdifferentiate and acquire a mesenchymal cell-like phenotype (Grisanti 1995). This transdifferentiation corresponds with decreased expression of cytokeratin 18, an epithelial marker; redistribution of the actin
cytoskeleton, and de novo expression of smooth muscle α-actin (Grisanti 1995). With continued passage *in vitro*, cultured porcine RPE cells are increasingly able to adhere to and exert tractional forces on the extracellular matrix (Grisanti 1997b). TGF-β₁, ₂ and ₃ were shown to inhibit the proliferation of the primary cultures of porcine RPE cells in a dose-dependent fashion but had no effect on the 10ᵗʰ passage RPE cells (Lee 2001).

Unlike RPE *in situ*, cultured RPE cells were found not to express CD95L using RT-PCR, western blotting, Rnase protection assay and flow cytometry (Kaestel 2001). This may have implications for ocular immune privilege.

Recently, immortalised RPE cells either arising spontaneously or produced by transfecting RPE cells with SV-40 large T antigen have been used (Nabi 1993, Dunn 1996). ARPE-19 is a spontaneously arising RPE cell line, which was produced by selective trypsinisation of the primary culture leading to cells with more uniform epitheloid morphology. ARPE-19 cells were shown to exhibit morphological polarisation when plated on laminin-coated transwell-COL filters as well as tight junction formation in medium with a low serum content (Dunn 1996). SV-40 immortalised RPE cell lines were shown to be positive for cytokeratin and to express ICAM-1 and MHC class I constitutively and could be induced to express VCAM-1 and class II MHC (Greenwood 1996). In addition, the immortalised RPE cells showed many other features of RPE *in vivo* such as expression of CD54, CD58 and CD59 and production of many growth factors. Unlike RPE cells *in vivo*, the immortalised cells failed to express CD95L, CD40 or CD48 (Kanuga 2002). Cultured transformed foetal human RPE cells were shown to preferentially express TGF-β₁ mRNA and some TGF-β₂ but no TGF-β₂. Conversely, the non-transformed cells predominantly secreted TGF-β₂ as is the case *in vivo* (Kvanta 1994).

Although cultured RPE cells and particularly RPE cell lines possess many of the characteristics of fresh RPE, it is apparent that there are fundamental differences between them. The heterogeneity amongst RPE cells and the fact that different populations of RPE cells may undertake different aspects of their diverse function are of note. This cellular diversity not represented *in vitro* may be responsible for differing and at times conflicting characteristics of RPE cells. Moreover presence or absence of other tissues such as the choroid, and the neural retina changes the phenotype and function of RPE cells. To circumvent the problem associated with the use of cultured RPE, in the following studies an ex-vivo model of posterior eyecup is used.
1.16 Research objectives and specific aims

As retinal transplantation becomes more feasible with better surgical techniques and the possibility of using stem cells, the assessment of the space into which the transplanted material is placed becomes increasingly important. Most studies performed have concentrated on the survival of the grafts placed in the subretinal space. Very little information is available on the mechanisms by which the subretinal space confers its immune privilege status. Moreover most of the information present has been gathered regarding the subretinal space of normal animals. There are scant studies that shed light on the state of the subretinal space in diseased animals and humans. Since the goal of transplantation is to return function to the eyes with retinal diseases and since, as demonstrated previously, the animals and humans with retinal diseases display alteration in the structure and integrity of the subretinal space, it is of utmost importance to ascertain how the subretinal space microenvironment is changed as the result of retinal disease and how this change affects immune privilege of the subretinal space.

The main objectives of my research are: 1) To assess the vulnerability of the monolayer of RPE to immune mediated attack, 2) to study the contribution of RPE to the immune privilege of the subretinal space by studying soluble factors produced by the monolayer of RPE and their effect on the cells and molecules of the immune system and 3) to investigate how diseased states change the immunosuppressive microenvironment of the subretinal space. Since the subretinal space faces the apical surface of RPE and since it was shown that the integrity of the monolayer of RPE is important in the immune privilege of the subretinal space, I have established an ex-vivo system of posterior eyecup that allows the RPE to be studied in a monolayer with their tight junctions intact and while in contact with the choroid.

Organ culture models have been used previously by Rosenstock et al. used RPE cup in bovine model to study phagocytosis (Rosenstock 1980). Wang and co-workers used superfused rat eyecup organ culture to assess contribution of various layers of the eye to production of plasminogen activator (Wang 1995). The organ culture model of posterior eyecup allows the cells to remain at least partially in their physiological states by remaining in contact with the choroid; to keep their polarity and hence their polar secretions of factors intact; and most importantly to remain as an intact monolayer with intact tight junctions.
Researchers studying the immune privilege of the anterior chamber use aqueous humour to investigate the immunomodulatory microenvironment of anterior chamber. There is no equivalent fluid available for the subretinal space. The posterior eyecup is incubated with serum free medium and the supernatant of these eyecups, after a defined period of time, are used as a partial representation of the interstitial fluid that bathes the subretinal space. Since the RPE are the only cells facing the eyecup, this supernatant is in effect the product of RPE cells. By studying the supernatant of RPE eyecup, the contribution of the RPE to the microenvironment of the subretinal space can be investigated.

1.16.1 The specific aims are

1. To assess the vulnerability of the RPE layer to disruption by cells of adaptive immunity.

2. To determine the effect of factors produced by the apical surface of the RPE on the cells of the innate and cellular adaptive immune system.

3. To identify factors present in the supernatant that maybe responsible for the effect of the supernatant on the immune cells.

4. To study the changes in the ability of RPE from animals with retinal diseases, either arising de novo or produced experimentally, to interact with the cells of the immune system.
Chapter 2 – Materials and Methods

2.1 Cell lines

Mink lung epithelial cells (Mv1Lu) were obtained from the American Tissue Culture Collection (CCL-64, ATCC, Rockville, MD, USA) and maintained in complete EMEM medium. EL4 and YAC-1 cells, mouse lymphoma cell lines were obtained from ATCC and grown in complete RPMI medium. RAW 264.7, a mouse monocytic leukaemia cell line, was obtained from ATCC and grown in complete RPMI medium.

2.2 Mice

Adult male BALB/c, C57BL/6, C57BL/6 gld/gld (B6.gld), C3H/HeN and DO11.10 mice aged 6-8 weeks were obtained from the animal facilities at the Schepens Eye Research Institute or from Taconic (Germantown, NY, USA) and Jackson Laboratories (Bar Harbor, ME, USA). Thrombospondin-1 knock out mice (TSP-1KO) were a generous gift from Dr. Jack Lawler (Beth Israel Deaconess Medical Center, Boston, MA, USA). TGF-β receptor II double negative (TGF-β RII DN) mice were kindly donated by Dr. Ron Gress (National Cancer Institute, Bethesda, MD, USA). Mice were kept in a common room of vivarium. All experimental procedures conformed to the ARVO Statement for the Use of Animals for Ophthalmic and Vision Research. Inoculations, injections and clinical examinations were performed under anaesthesia induced by intraperitoneal injection of ketamin (Ketalar; Parke Davis, Paramus, NJ, USA) at 0.075 mg/g of body weight, and xylazine (Rompun, Phoenix Pharmaceuticals, St. Joseph, MO, USA) at 0.006 mg/g of body weight. Enucleation and removal of spleen and lymph nodes were performed after the animals were sacrificed by cervical dislocation.

2.3 Preparation of posterior eyecups

Eyes from C57BL/6, C3H/HeN, C57BL/6 gld/gld (B6.gld) and thrombospondin-1 knockout mice (TSP-1KO) were enucleated and placed in Ca++/Mg++ free HBSS on ice for thirty minutes. They were then removed and the muscles, connective tissue and conjunctiva were excised with micro scissors. A circumferential incision was performed below the level
of pars plana to ensure complete removal of the ciliary body and the anterior segment including the cornea, iris, ciliary body and the lens were removed. The remaining tissue was placed in 0.01 units per ml of chondroitinase ABC (Yao 1992) for thirty minutes at 37°C, then placed on ice and washed three times in HBSS. Neural retina was gently lifted off the RPE layer by microsurgical forceps. Posterior eyecups consisting of sclera, choroid and a healthy monolayer of RPE were placed in individual wells of microculture plate (S plate, Nunc Brand Products, Nalge Nunc International Corporation, Naperville, IL, USA) for further experiments. To ascertain that RPE in these eyecups retain their monolayer status, the eyecups were fixed in 10% formalin, embedded in methacrylate, sectioned and stained with H&E. The sections were then viewed with light microscopy. Figure 2.1A depicts the continuous monolayer of RPE with normal cytostructure. Since the presence of tight junctions is essential to the integrity of RPE as a monolayer, the posterior eyecups were stained with ZO1 antibody and viewed by confocal microscopy. The tight junctions remain intact as evidenced by the level of ZO-1 expression (Figure 2.1B).
Figure 2.1: Photomicrograph of posterior eyecups from C57BL/6 mice

Posterior eyecups were prepared from eyes of C57BL/6 mice.

A. The eyecups were fixed with 10% formalin, embedded in methacrylate, sectioned, stained with H&E and viewed by light microscopy. A continuous layer of RPE with regular spacing and normal morphology is present.

B. Prepared eyecups were stained with ZO-1 antibody to depict tight junctions viewed with Confocal Microscopy. RPE strongly exhibits ZO-1 staining.
2.4 DO11.10 T cell proliferation assay

The proliferation assay was performed using the lymph node cells from DO11.10 transgenic mice. DO11.10 mice were sacrificed and their lymph nodes were removed. 4 x 10^5 DO11.10 lymph node cells in volume of 25 μl were placed in wells of a 96-well round bottom plate (Costar, Fisher Scientific, Pittsburgh, PA, USA). Supernatant of RPE eyecups (SN) were collected after the eyecup was cultured with Serum Free Medium (SFM) (Appendix I) for various time intervals. 50 μl of 1:10 diluted SN of RPE per well was incubated with the DO11.10 lymph node cells and of 50μg/ml ovalbumin (OVA) (Sigma, St. Louis, MO, USA) in volume 25μl for 96 hours at 37°C. 0.5 μCi of ^3H-thymidine (Nen Life Sciences Products, Boston, MA, USA) was added to each well on day 4 and incubated overnight. Thymidine uptake was measured as a function of T cell proliferation. As a positive control, some wells contained DO11.10 lymph node cells with OVA but without the SN of the RPE eyecup to assess total T cell activation. Wells with only DO11.10 lymph node cells and SFM served as negative control for background T cell activation. Three wells were used for each time point and the experiment was repeated three times.

2.5 Preparation of allo-sensitised effector T cells

BALB/c mice were immunised in the flank by injecting 1x 10^7 C57BL/6 splenocytes on day zero. They were sacrificed a week later and their spleens were removed and rendered into a single cell suspension by passing them gently through a mesh. Red blood cells were eliminated using red blood cell-lysing solution. The splenocytes were co cultured at a ratio of 5:1 with irradiated (2000 rad) naïve lymphocytes from C57BL/6 mice for 5 days in complete RPMI medium (Appendix). After five days the cells were washed three times in HBSS. The live cells were counted and used as BALB/c anti C57BL/6 as effector cells in subsequent experiments. As control, C57BL/6 anti BALB/c effectors were produced in a similar manner.

2.6 Assay for cytotoxic activity

BALB/c anti C57BL/6 effector cells were tested for cell-mediated cytotoxicity in a standard 4-hour ^51Cr-release assay. EL4 cells, a lymphoma cell line derived from a C57BL/6 strain, were used as target cells. They were labelled with 300 μCi Na_2^{51}CrO_4 (New England Nuclear, Boston, MA, USA) per 3 x 10^6 cells for 2 hours in the water bath at 37°C.
labelling EL4 cells were washed three times and placed in the microtitre plate at concentration of 2 x 10^4 per well. The BALB/c anti C57BL/6 effector cells and as a control, the C57BL/6 anti BALB/c effector cells were placed into the wells of microtitre plate at the following effector to target ratio (E: T): 100:1, 10:1, 2:1 and 1:1. Culture plates were centrifuged at 1000 rpm for 5 minutes and incubated for 4 hours 37°C. After incubation, 25 μl of the supernatant was removed and counted for radioactivity. The percentage of specific chromium release was calculated using the standard formula. The spontaneous release of ^{51}Cr from the target cells was determined by counting the supernatant from three wells containing only EL4 target cells and no effector cells. The total release was determined by measuring chromium released from the supernatant of 3 wells containing the EL4 cells and 1N HCl. Spontaneous release of chromium never exceeded 20% of the total release.

2.7 Injection into the subretinal space

Subretinal space injections were performed according to the procedure of Whiteley et al. (Whitely 2001) on anaesthetised animals that, in addition, received topical proparacain to anaesthetise the ocular surface and tropecamide 1% to dilate the right pupil. Injection into the subretinal space was performed using very fine, bevelled pulled glass micropipettes that was connected to a 10 μl Hamilton syringe via a fine polyethylene tube. The entire apparatus was filled with HBSS, but an airlock was produced prior to the volume to be injected preventing dilution of the injected material. The bore of the glass needle was coated with sigma-coat (Sigma) to prevent adherence of cells. The injections were made under direct visualisation via a trans-scleral approach through the peripheral retina using a binocular surgical microscope and a cover slip held on the cornea. The glass needle was advanced carefully until it reached the subretinal space where cells in a volume of 1μl were injected.

2.8 Assessment of immune deviation

Delayed hypersensitivity (DH), for OVA antigen was evaluated 14 days after injection of OVA. Ear swelling analysis was performed seven days later. Delayed hypersensitivity (DH), for OVA antigen was measured based on ear swelling as previously described (Jiang 1994, Wenkel 1998). Experimental animals received either 50 μg of OVA into the subretinal space or the anterior chamber. Seven days later, the mice were subcutaneously immunised.
with 100 μg of OVA and complete Freund’s adjuvant (CFA) (Difco, Detroit, MI, USA) in the ratio of 1:1. On day 14, 200 μg of OVA in 10 μl of HBSS were injected into the right ear pinnae of all mice. The left ear served as the untreated control. Both ear pinnae were measured immediately before and 24 and 48 hours after the ear injection with an engineer’s micrometer (Mitutoyo, Tokyo, Japan). The measurements were performed in triplicates. Results were expressed as specific ear swelling = (24-hour measurement - 0-hour measurement in the experimental ear - 24-hour measurement in the negative control ear) x 10^-3 mm. A two-tailed Student’s t-test was used with significance assumed at p < 0.05.

2.9 TGF-β bioassay

The concentration of TGF-β in the supernatant of the RPE was measured as previously described for quantification in the aqueous humour using the standard mink lung epithelial cell proliferation inhibition assay (Cousins 1991). Mv1Lu cells from sub-confluent cultures were incubated with a serial dilution of unmodified or acid treated SN of the RPE eyecup in 96-well flat bottom microtitre plates (Costar, Fisher Scientific, Pittsburgh, PA, USA). Media used was EMEM (Gibco BRL, Gaithersburg, MD, USA) supplemented with 0.05% foetal calf serum. Cultures were incubated for 20 hours at 37°C. At this time 0.5 μCi of ³H-thymidine was added to each culture and four hours later the amount of incorporated label was measured by scintillation counting. Concentration of TGF-β was determined by comparing the half-maximal inhibition of the cultures treated with diluted supernatant of RPE eyecups with a standard curve from the cell cultures treated with known amounts of TGF-β. Specificity of the assay was monitored by treating the cells with the SN of the RPE eyecups in the presence of neutralising anti-TGF-β antibody (R&D Systems, Minneapolis, MN, USA). The isotype of TGF-β present was determined by using anti-TGF-β₁ and anti-TGF-β₂ antibodies (R&D Systems) at a concentration 10 times the specified 50% neutralising dose. The antibody mixtures were incubated for 1 hour at 37°C with the SN of the RPE eyecup prior to experimentation.
2.10 IFN-γ ELISA

IFN-γ production was assayed by using sandwich ELISA. The wells of 96-well microtitre plate (Falcon, Oxnard, CA, USA) was coated with capturing mAb to IFN-γ (Pharmingen, San Diego, CA, USA) and incubated overnight at 4°C after which it was blocked with PBS containing 1% BSA and washed with the wash buffer (Appendix). Sample and standard recombinant IFN-γ were applied to the plate and incubated for 3 h at room temperature. The plate was washed and biotinlated detecting antibody (Pharmingen, San Diego, CA, USA) to the cytokine was added, incubated for 1 h and was washed. Streptavidin-β-galactosidase (100 ml Gibco BRL, Gaithersburg, MD, USA) was added to the wells, incubated for 30 min, washed and the substrate chlorophenyl-red-β-D-galactoside (Calbiochem, San Diego, CA, USA) was added to the wells. A standard ELISA plate reader was used to read the optical density of the colour change at a wavelength of 570 nm. The concentration of cytokine in the sample was calculated from a standard based on the optical density of the curve made from the optical density versus the corresponding concentration of standard cytokine.

2.11 Somatostatin ELISA

The concentration of somatostatin (SOM) in the SN of the RPE eyecups was measured using a competitive ELISA method developed by the laboratory of Dr. Andrew Taylor (Taylor 2003). A 96 well flat bottom plate (Corning, Corning, NY, USA) was coated with 1:500 dilution of anti-Rb IgG (Sigma Chemical, St. Louis, MO, USA) overnight. The plate was then blocked and incubated with a 1/400 dilution of anti-SOM antibody. Diluted supernatant of the RPE eyecup were mixed with 2ng/ml of biotinylated-SOM and added to the wells. To prepare the standard curve, known quantities of SOM protein (10-0.003 ng/ml) were mixed with the biotinylated-SOM. 1% BSA (Sigma) in 0.1 M PBS (PBS-BSA) was used to block, wash the wells and dilute the antibodies. The plate was incubated for two hours at room temperature and washed. Diluted (1:1000) strepavidin-β galactosidase (Gibco BRL, Gaithersburg, MD, USA) was added to the wells and the plate was incubated for 30 minutes at room temperature. After washing, the substrate chlorophenyl-red-β-d-galactoside (Gibco BRL) was added and the optical density of the colour change was read 1 hour later with a Quant microplate spectrophotometer (Bio-Tek, Winooski, VT, USA). An equation fitted to
the polynomial regression of known SOM concentrations was used to calculate the concentration of SOM in the SN of the RPE eyecup from the samples’ optical densities. The sensitivity of the competitive ELISA was down to 3 pg/ml.

2.12 RNA isolation

Total RNA was isolated from RPE cells harvested from eyecups and from confluent second passage of cultured Monodisperse RPE, using RNA STAT-60 kit (Tel-Test, Inc., Friendswood, TX) according to the manufacturer’s instructions provided. This kit utilises a single-step method by acid guanidinium thiocyanate-phenol-chloroform extraction.

2.13 cDNA synthesis

1 μg of total RNA from each group was added to the microcentrifuge tube and 2 μl of 50 μM oligo primer stock was added. The volume adjusted to 13 μl with water. The reaction was incubated for 10 minutes at 70°C. After a brief centrifugation, 7 μl of master mix was added to the mixture (4 μl of 5x first-strand buffer, 1.0 μl of RNase inhibitor, 1.0 μl dNTP mix, 0.9 μl of water, 0.1 μl of MMLV-RT). The mixtures were incubated at room temperature for 10 minutes followed by 60 minutes incubation at 37°C. The reaction was heated to 90°C for 5 minutes to inactivate RT. Finally it was chilled on ice for 4 minutes and stored at 4°C.

2.14 RT-PCR

cDNA was synthesised by reverse transcribing RNA using random hexamers and AMV RT (Promega). For PCR amplification of thrombospondin cDNAs were amplified using primers as listed below: (5’ to 3’ sequences) F-TSP, GTT CGT CGG AAG GAT TGT TA, R-TSP, TCT ATT CCA ATG GCA ACG AG (733 bp); and for somatostatin F-ppSOM, TGG CTT TGG GCG GTG TCA, and R-ppSOM, CAG CCA GCT TTG CGT TCC (265 bp). Primers for GAPDH were, F-GAPH, GGTGAAGGTCGGTGTGAACGGA; R-GAPDH, TGTTAGTGGGGTCTCGCTCCTG (245 bp). TSP: Intron spanning primers for the specific amplification of selected genes were designed using gene sequences from the public database and software Oligo Primer Analysis software 6.0 (Molecular Biology Insights, Inc., Plymouth, MN, USA). PCR reactions were performed in a 50 μl amplification mixture
containing 1X polymerase buffer, 2.5 mM MgCl₂, 0.2 mM each dNTP, 1 µM of forward and reverse primers, 1.25 U Taq polymerase (Perkin Elmer). Following PCR thermal profile was performed in a thermal cycler (GeneAmp PCR System 2400, Perkin Elmer). 1 cycle: (5 min at 94°C, 5 min at 60°C), 40 cycles: (2 min at 72°C, 1 min at 94°C, 1 min at 58°C), 1 cycle: (10 min at 72°C, hold at 4°C) PCR products were separated by 1.5% agarose gel electrophoresis.

2.15 Induction of experimental autoimmune uveoretinitis (EAU)

Human IRBP peptide 1-20 (GPTHLFQPSLVLDMAKVLLD) was synthesised by Invitrogen life technologies (Carlsbad, CA, USA). Complete Freund’s adjuvant (CFA), incomplete Freund’s adjuvant (IFA), and *Mycobacterium tuberculosis* H37RA were purchased from Difco Laboratories (Detroit, MI, USA). Purified *Bordetella pertussis* toxin (PTX) was from Sigma Chemical (St. Louis, MO, USA).

To induce EAU in C57BL/6 and TSP-1 knockout mice (H-2ᵇ) mice, they were immunised subcutaneously with 200 µg of IRBPp 1-20 emulsified with CFA that contains 6.0 (final 3.0) mg/ml of *Mycobacterium tuberculosis* H37RA. The mice were also given 0.1 µg of PTX intraperitoneally as additional adjuvant, reported elsewhere (Taylor 2002). The disease severity was clinically assessed by fundus examination that was performed under Ketamine and Xylazine anesthesia (Phoenix Pharmaceutical, St Joseph, MO, USA). The pupils were dilated with 1% tropicamide ophthalmic solution (Akorn, Buffalo Grove, IL, USA) before the examination. The clinical scoring of EAU was based on vessel dilatation, number of vessel white focal lesions, and the extent of retinal vessel exudates, haemorrhage, and detachment. The clinical severity was graded on 0 to 5 scales as described previously (Taylor 2002, Namba 2000, Namba 2002, Kitaichi 2002). The difference between two mean clinical scores was statistically analyzed by the non-parametric Mann-Whitney U test for the comparison of two independent populations. Differences were called significant when the *P* value of a statistical analysis was equal or less than 0.05.

2.16 Neutrophil activation

C57BL/6 mice received intraperitoneal injection of 9% Casein (in 0.9% saline) 18 hours and again 3 hours prior to harvesting the neutrophils. At the time of the harvesting, the
animals were sacrificed and the skin over the abdomen washed thoroughly with alcohol. The skin was then incised revealing sealed peritoneum. 5 ml of cold sterile PBS with 0.02% EDTA was injected into the peritoneal cavity. The fluid was mobilised within the cavity and withdrawn. This procedure was repeated twice yielding 10 ml of PBS containing neutrophils. The solution was spun and washed three times and the cells were counted. The cells in the volume of 1 ml were added to 9ml mixture of Percoll (9ml of Percoll and 1 ml of 10XPBS) (Pharmecia Biotech, Piscataway, NJ). The mixture was ultracentrifuged for 20 minutes at 60650 x g and 4°C. The neutrophils were collected from the second opaque layer, washed with PBS and checked for purity and viability by H&E staining of cytospin preparation. The neutrophils were placed in serum free medium (Appendix) and used in neutrophil activation assay.

2.17 IL-1β ELISA

IL-1β production was assayed by using sandwich ELISA. In brief, a 96-well microtitre plate was coated with capturing mAb to IL-1β (Cytosets Paired Antibody Assay System, Biosource International, Camarillo, CA, USA) and incubated overnight at 4°C after which it was blocked with PBS containing 1% BSA and Tween-20 for 2 hours at room temperature. The plate was then washed with wash buffer (Appendix) and samples and standard recombinant IL-1β were applied to the plate and incubated for 1:30 h at room temperature. The plate was washed and biotinylated detecting antibody to the cytokine (Cytosets Paired Antibody Assay System) at concentration of 0.25 μg/ml was added, incubated for 1 h and was washed. Streptavidin-β HRP (Cytosets Paired Antibody Assay System) was added to the wells, incubated for 45 min, washed and the substrate TMB (Immunopure TMB Substrate Kit, Pierce, Rockford, IL, USA) was then added to the wells for 30 minutes. The reaction was stopped by the addition of 1.0N H2SO4. A standard ELISA plate reader was used to read the optical density of the colour change at a wavelength of 450 nm. The concentration of cytokine in the sample was calculated from a standard based on the optical density of the curve made from the optical density versus the corresponding concentration of standard cytokine.
2.18 Nitric oxide (NO) assay

Accumulation of nitrite in the culture supernatant of LPS-activated RAW cell line was assayed as an indicator on nitric oxide production. RAW cells, in phenol-free DMEM supplemented with 10% foetal calf serum (Hyclone, Logan, UT, USA), were placed in the wells of flat-bottom 96-well plate at the concentration of $1.5 \times 10^5$ cells per well for 2 hours at 37°C. The medium was removed and was replaced with 100 μl of phenol-red free DMEM with 0.5% of FCS. Experimental wells contained 50 μl of 1μg/ml of LPS and 24 hour diluted SN of RPE eyecup. In order to ascertain the role that TGF-β and SOM play, some experimental wells also received anti-TGF-β or anti-SOM antibodies. 2 μg/ml of recombinant SOM was added to some wells, with or without the addition of LPS, to confirm the role of SOM in controlling NO production. Wells containing RAW cells and LPS, acted as positive controls and those containing only RAW cells served as negative control. The cultures were incubated for 24 hours at 37°C. The supernatant was assayed for NO by mixing 100 μl of supernatant with 100 μl Griess reagent (Green 1982) (1% sulfanilamide-0.1% naphthylethylene diamine dihydrochloride in 2% H$_3$PO$_4$) in a 96-well plate. After 15 minutes of incubation at room temperature, the plate was read at 550 nm using an electroimmunoassay plate reader. The concentration of nitrite in the culture supernatant was determined from a standard curve of known sodium nitrite concentrations (100-0.003 μM).

2.19 NK cells cytotoxic assay

Natural killer cells were tested for cell-mediated cytotoxicity in a standard 4-hour $^{51}$Cr-release assay. YAC-1 cells, a murine lymphoma cell line, were used as target cells. They were labelled with 300 μCi Na$_2$ $^{51}$CrO$_4$ (New England Nuclear, Boston, MA, USA) per 3 x10$^6$ cells for 2 hours in the water bath at 37°C. Thereafter, YAC-1 cells were washed three times and placed in the microtitre plate at concentration of $1 \times 10^4$ per well. The splenocytes were added into the wells of microtitre plate at the following effector to target ratio (E: T): 250:1, 100:1 and 50:1. Culture plates were centrifuged at 1000 rpm for 5 minutes and incubated for 4 hours 37°C. After incubation, 25 μl of the supernatant was removed and counted for radioactivity. The percentage of specific chromium release was calculated using the standard formula. The spontaneous release of $^{51}$Cr from the target cells was determined by counting the supernatant from three wells containing only YAC-1 target cells and no effector cells. The
total release was determined by measuring chromium released from the supernatant of 3 wells containing the YAC-1 cells and 1N HCl. Spontaneous release of chromium never exceeded 20% of the total release.

2.20 Anti-CD3 T cell activation assay

Spleen and lymph nodes were removed from C57BL/6 or TGF-β RII DN mice and naïve T cells were purified by using CD3-enrichment columns (R&D Systems). 4 x 10^5 T cells were resuspended in serum free medium per well and added to the wells of a 96-well round bottom plates at the volume of 25 μl per well (Corning). Anti-TCR antibody (2C11; 1μg/ml) and 50 μl of 1:10 diluted SN of RPE eyecups prepared from the eyes of C57BL/6 were also added to the experimental wells. Some of the culture wells were incubated for 24 hours, after which time 0.5 μCi of ^3H-thymidine was added to the wells and the cultures were incubated for a further 24 hours. Thymidine uptake was measured as a function of T cell proliferation. Other culture wells were incubated for 48 hours, after which time their supernatants were removed and used in IFN-γ ELISA assay to measure the amount of IFN-γ produced by T cells. Some wells, serving as positive controls, only contained T cells activated by anti-CD3 antibody, and other wells, serving as negative controls, only contained T cells without the RPE SNs and anti-CD3 stimulation.

2.21 IFN-γ treatment and histology

Class I expression was stimulated in posterior eyecups by exposing the tissues in vitro to IFN-γ (recombinant mouse IFN-γ, R & D systems) for 4 or 12 hours, prior to testing the effect of specifically sensitised T cells on RPE lysis. The eyecups were then placed on microscope slides and treated with FITC conjugated anti-mouse H-2kB (pharmingen) to assess the level of class I expression. The eyecups were subsequently stained with propidium iodide and acridine orange to assess the survival of the RPE cells.
Chapter 3 – RPE as a target of adaptive cellular immunity

3.1 Introduction

Neural retina and RPE are vastly different types of tissues, and each confronts the experimentalist with a unique set of barriers to successful transplantation. It has been demonstrated that RPE as a tissue expresses class I alloantigens encoded within the murine major histocompatibility complex (MHC, H-2) (Wang 1987) and it is presumed that RPE also displays on these class I molecules, peptides derived from proteins encoded by the MHC and minor histocompatibility antigens. Under normal circumstances, the RPE expresses no class II MHC molecules, although the expression of these molecules can be induced by exposure to IFN-γ (Osusky 1997). Thus, in principle, allografts of monodisperse RPE and intact RPE monolayers should represent suitable targets for CD8+ T cells of the cytotoxic type.

3.1.1 T cells: Ontogeny and differentiation into effector subsets

T lymphocytes originate from a pluripotent stem cell in the bone marrow and become committed to becoming a T cell prior to leaving. T cell precursors (pre T cells) travel to the cortex of the thymus as CD4+CD8+ lymphoblasts and then differentiate into CD4+ or CD8+ T cells. These naïve T cells then disseminate into secondary lymphoid organs (lymph nodes, spleen and Peyer’s patches). Entrance of naïve T cells into secondary lymphoid organ is a complex, multi-step process that is dependent on adhesion molecules and chemokines. The 2 major subtypes of T cells are divided based on functional studies: CD4+ T cells or T helper cells (Th) provide helper function for antibody responses, are responsible for DH, and regulate the activity of effector cells; and CD8+ effector cells that mediate cytotoxicity and perform regulatory and suppressor action in immune privilege. CD4 T cell recognise foreign antigen in association with class II MHC and CD8+ cells in the context of class I.

3.1.2 CD8 positive T cells

Naïve CD8+ T cells recognise target antigens in association with class I MHC (signal one) and after co-stimulation (signal two), proliferate and differentiate into effector CD8+ cells. Co-stimulation is either provided by professional APCs (B7 family molecules)
presenting the antigen or through production of cytokines, by CD4\(^+\) T helper cells.
Recognition of the MHC-associated peptide on the target cells induced clustering of T cell receptors (TCR) to the site of contact and leads to activation of CTLs. Activated CTLs undergo cytostructural changes that results in exocytosis of the content of CTL granules, mainly perforin and granzymes, on to cell surface of the target cell leading to the target cells death. CTLs also express FasL that after cross-linking Fas protein on target cells leads to lysis of the target cells.

At present, there is no direct information in the literature concerning the vulnerability of RPE to destruction by allo-specific cytotoxic T cells (Tc). RPE grafts, as single cell suspensions of cultured cells, and as intact sheets prepared from neonatal mouse eyes, have been implanted into the anterior chamber, the vitreous cavity, and even into the subretinal space of mouse eyes (Jiang 1994, Grisanti 1997). Since all these intraocular compartments display immune privilege, interpretation of the fate of allografts of RPE, is complicated by the contribution to graft survival made by the site itself. Wenkel and Streilein addressed this issue when they transplanted sheets of allogeneic neonatal RPE tissue beneath the capsule of the kidney – a non-immune privileged site (Wenkel 2000). They reported that allogeneic as well as syngeneic RPE grafts survived beneath the kidney capsule – these transplants showed no gross or histological evidence of rejection for at least 8 weeks after implantation. This finding offers formal proof that neonatal RPE tissue has the inherent property of immune privilege. These investigators also showed that the privileged status of RPE allografts could be aborted if the grafts failed to express CD95 ligand (CD95L). Thus, similar to the corneal endothelium, and to the testes, RPE functions as an immune privileged tissue in part through the constitutive expression of CD95L (Bellgrau 1995, Stuart 1997).

RPE and corneal endothelium share similar expression patterns for the MHC-encoded alloantigens, and it is relevant that orthotopic allografts of cornea owe their high rate of survival in normal, untreated mouse eyes to the constitutive expression of CD95L (Stuart 1997, Yamagami 1997). At present, it is not technically feasible to transplant sheets of RPE orthotopically into the subretinal space of mouse eyes. For this reason, the following series of studies was embarked upon to determine the vulnerability of RPE to immune destruction by Tc using strategies that do not involve orthotopic transplantation of the tissue.
3.2 Results

3.2.1 Vulnerability of RPE cells to immune destruction by effector T cells placed subretinally

Primed H-2<sup>b</sup>-specific cytotoxic T cells were generated by immunising BALB/c mice subcutaneously with C57BL/6 spleen cells. One week later spleen cells were harvested from these mice and re-stimulated <i>in vitro</i> with X-irradiated C57BL/6 spleen cells. After 5 days, the responding lymphocytes were harvested and assayed for their capacity to lyse EL-4 cells, a target tumour cell line derived from the C57BL/6 mouse strain. Control effector cells were prepared similarly by immunising C57BL/6 mice with BALB/c spleen cells. As revealed in Figure 3.1, BALB/c effector cells lysed target EL4 cells expressing H-2<sup>b</sup> class I alloantigens. EL4 cells were not lysed by C57BL/6 effector cells, demonstrating the specificity of the cytotoxic attack.

Effector cells prepared in this manner were injected (10,000 cells per 1 μl inoculum) into the subretinal space of eyes of normal C57BL/6 recipients. As controls, eyes of some mice received a 1 μl inoculum of PBS alone into the subretinal space. In one set of recipients, the eyes were examined clinically using fundoscopy at 2, 5 and 14 days post-injection. In another set, injected eyes were removed at 1, 6, 24, 28 h, and at 14 days post-injection; these eyes were prepared and sectioned for histological examination.

Clinical examination after 2 days revealed that the retina was oedematous in an area of one disc diameter (diameter of the optic nerve head), at the site of injection. This oedematous appearance was detected irrespective of the contents of the subretinal injection. At the same time, the anterior chamber was normal, the lens was clear, and there was no evidence of intraocular inflammation. These findings indicate that there is a minimal amount of trauma associated with subretinal injections of this type, and that the focus of this response is restricted to the site of injection. Subsequently, eyes that received a subretinal injection of BALB/c anti-C57BL/6 lymphoid cells, a distinct pallor developed at the injection site at 5 days post-injection that was readily distinguishable from surrounding tissue and was approximately two disc diameters in size. This defect in the RPE layer was still present when these eyes were examined at 14 days post-injection, but had not changed in size during this time interval. Importantly, no area of pallor was detected in the retinas of eyes that received
injections of control lymphoid cells. Thus, injection of specifically sensitised lymphoid cells containing Tc created a circumscribed defect in the RPE monolayer at the site of injection.
Figure 3.1: BALB/c spleen cells primed *in vivo* and stimulated *in vitro* against C57BL/6 alloantigens are able to kill EL4 cells.

BALB/c spleen cells primed against C57BL/6 targets were incubated with $^{51}$Cr labelled EL4 cells, a C57BL/6 origin lymphoma cells, for 4 hours at 37°C. As a control, C57BL/6 splenocytes primed against BALB/c targets were similarly incubated with EL4 cells. BALB/c anti-C57BL/6 effector cells were able to lyse EL4 cells in effective to target ratios dependent manner. C57BL/6 anti BALB/c lymphoid cells were unable to lyse EL4 cells at any E: T ratio.
% death

E : T

- BALB/c anti C57BL/6
- C57BL/6 anti BALB/c
Once established, this lesion failed to expand in size. Histological examination of eyes receiving effector cell injections supported these observations (Figure 3.2). In eyes that received effector cells injections, lymphoid cells were detected in the subretinal space at 1 and 6 h (Figure 3.2A and B). Among eyes that received control cells, no lymphoid cells were seen thereafter, but among eyes that received BALB/c anti-C57BL/6 effector cells, inflammatory cells persisted at the injection site through 24 hours post injection (Figure 3.2C).

At 48 hours post injection, all eyes contained oedema fluid in the subretinal space and within the retina itself around the site of injection. When examined at later intervals, neither inflammatory cells nor oedema was detected at the injection site, and the RPE layer was intact (Figure 3.3A and B). By contrast, at 5 days the RPE layer was disrupted at the site of injection of BALB/c anti-C57BL/6 cells. Some RPE cells appeared vacuolated and had lifted off Bruch’s membrane (Figure 3.4A). At 14 days post-injection, the injection site was devoid of healthy RPE cells, and there was a sharp perimeter to the lesion formed by healthy-appearing RPE (Figure 3.4B and C). At no time were inflammatory cells found within the neural retina, nor was the photoreceptor layer of the retina damaged.

Together, the results of these experiments indicate that specifically sensitised effector lymphoid cells containing Tc are able to attack and destroy RPE at the precise site where they are injected but that the damage remains confined to this site, with little evidence to suggest that the effector cells can systematically and sequentially destroy adjacent RPE cells.
Figure 3.2: Histological appearance of subretinal space injection site of effector lymphocytes in C57BL/6 eyes

10,000 BALB/c anti C57BL/6 effector cells were injected into the subretinal space of adult C57BL/6 mice. One hour, 24 hours and 48 hours later the animals were sacrificed, their eyes were enucleated, placed in 10% formalin, embedded in methacrylate, sectioned, stained with H&E and viewed with light microscopy.

A. Arrow shows the injection site and the bleb produced around the site of injection.

B. Magnified view (X60) of the injection site reveals numerous lymphocytes and a few apoptotic cells. A similar image is observed when C57BL/6 anti BALB/c splenocytes were injected into the subretinal space of C57BL/6 mice.

C. This section is produced from the eyes receiving BALB/c anti C57BL/6 splenocytes 24 hours post injection. The arrow demonstrates the injection site. A retinal detachment was evident throughout the length of the retina yet few lymphoid cells are dispersed throughout the subretinal space. None of the injected cells was found at 24 h in other control.
Figure 3.2
Figure 3.3: Histological appearance of RPE and retina around the site of injection of C57BL/6 effector lymphocytes in C57BL/6 eyes

A and B. Control C57BL/6 effector cells sensitised to BALB/c alloantigen were injected into the subretinal space of adult C57BL/6 mice. Two weeks later the eyes were enucleated and prepared for histology. The RPE layer is intact and the retina has normal cytostructure. Diagram B is magnified (X60) to show the RPE cell nuclei. No inflammatory cells are visible.
Figure 3.3

A
Ganglion cell layer

Inner nuclear layer

Outer nuclear layer

Photoreceptor outer segment

RPE

Choroid

X20

B

Outer nuclear layer

Photoreceptor outer segment

RPE

Choroid

Sclera

X60
Figure 3.4: Histological appearance of RPE and retina around the site of injection of BALB/c effector lymphocytes in C57BL/6 eyes

A. This section, produced from eyes enucleated 5 days after receiving BALB/c effector cells primed against C57BL/6 antigens, depicts vacuoles within the damaged RPE segment and abnormalities in cytostructure of RPE cells.

B. and C. These sections were produced from eyes enucleated 14 days after receiving BALB/c effectors against C57BL/6 alloantigens. Figure B demonstrates the circumscribed extent of damage to the RPE layer (between the 2 arrows) flanked by normal RPE. Figure C depicts the loss of RPE cell nuclei and extensive disorganisation of cells within the damaged portion.
3.2.2 Vulnerability of CD95 ligand deficient RPE to immune destruction by effector T cells placed subretinally.

Since RPE express CD95L constitutively, and since CD95L expression on layers of allogeneic neonatal RPE implanted beneath the kidney capsule protects these grafts from immune destruction (Wenkel 2000), the next focus for the experiments was to examine whether enhanced destruction of RPE might occur when specifically sensitised lymphoid cells are injected into the subretinal space of B6.gld mice that lack expression of a functional CD95L molecule. C57BL/6-primed lymphoid cells that were re-stimulated in vitro were injected into the subretinal space of B6.gld mice, and the injected eyes were examined clinically and histologically as described above. As revealed in Figure 3.5, the pattern of clinical and histological findings in CD95L-deficient eyes that received injections of specifically sensitised and irrelevantly-sensitised lymphoid cells was identical to that found in normal eyes. In particular, the initial RPE lesion, its evolution, and final extent were no different in CD95L-deficient mice than in wild type eyes. Thus, expression of CD95L cannot explain the failure of specifically sensitised lymphoid cells injected into the subretinal space to create circumferentially expanding lesions in which the potential for larger and larger numbers of RPE cells to be destroyed is not realised.
Figure 3.5: Histological appearance of subretinal space injection site of effector lymphocytes in B6.gld eyes

Sections are from eyes of B6.gld mice enucleated 5 days (A) and 14 days (B and C) after receiving BALB/c effectors against C57BL/6 alloantigens. Well-circumscribed areas of vacuolation are visible at 5 days leading to the loss of the RPE layer around the site of injection at 14 days post injection.
3.2.3 Vulnerability of RPE cells to immune destruction by allogeneic effector T cells *in vitro*

The results of the experiments described to this point suggest that the resident RPE *in vivo* is highly resistant to the destructive potential of primed cytotoxic T cells. Since the putative effector cells were injected into the subretinal space, an immune privileged site, the possibility exists that properties of the site itself limited the lytic capacity of the injected cells. To circumvent this problem, a posterior eyecup was created by enucleating eyes from normal C57BL/6 mice by first excising away the anterior segment (cornea, conjunctiva, lens, iris, and ciliary body). The neural retina was then gently pulled away following enzymatic treatment, leaving behind an intact layer of RPE resting on Bruch's membrane, the choroid, and the posterior sclera. These eyecups were placed, RPE layer up, in microculture plates.

Effector T cell suspensions prepared from BALB/c and C57BL/6 donors as described above were layered (10,000 cells per 10 μl) gently on top of the RPE layer and incubated for 4 hours. The lymphoid cells were then rinsed away and the RPE layer of the eyecups was assessed for viability by staining with propidium iodide and acridine orange, transferring to cover slips, and inspection by confocal microscopy. The results, displayed in Figure 3.6A, reveal that allogeneic RPE in posterior eyecups were impervious to lysis by Tc, and that they were equally resistant to non-specific lysis by immunologically irrelevant, but activated lymphoid cells. Similar experiments were performed with posterior eyecups prepared from B6.gld donors. Once again (Figure 3.6B), BALB/c effector cells proved to be incapable of lysing CD95L-deficient RPE.

One possible explanation for the invulnerability of RPE in the posterior eyecups to lysis by Tc is that the constitutive level of the MHC class I expression is insufficient to trigger effector cells. To address this point, class I expression was stimulated in posterior eyecups by exposing the tissues *in vitro* to IFN-γ for 4 or 12 hours, prior to testing the effect of specifically sensitised T cells on RPE lysis. The expression of class I MHC molecules was significantly increased on RPE cells in eyecups exposed to IFN-γ for 12, but not, 4 hours (3.7). As revealed in Figure 3.8C, RPE in posterior eyecups treated with IFN-γ for 12 hours showed a higher uptake of propidium iodide upon exposure to Tc than did untreated RPE. A comparatively small specific RPE cell death was detected in eyecups treated with IFN-γ for only 4 hours (Figure 3.8B). These results suggest that low-level expression of the MHC class
I molecules is at least one factor that renders RPE relatively resistant to lysis by allo-specific Tc.
Figure 3.6: Confocal appearance of RPE layers from eyes of C57BL/6 and B6.gld mice after incubation with effector T cells

Posterior eyecups were produced from enucleated eyes of adult C57BL/6 and B6.gld mice and were placed in individual wells of a microculture plate. BALB/c anti C57BL/6 effector T cells were layered into the eyecups. After incubation for 4 hours, the eyecups were stained with propidium iodide (PI) and acridine orange (AO). The cups were mounted on glass slides, covered with glass cover slips and observed with Confocal Microscopy.

A. C57BL/6 eyecup
B. B6.gld eyecup.

Both show the intact monolayer of retinal pigment epithelium bearing only the AO staining. A few yellow spots (combination of PI and AO) shown is from dead splenocytes that were left after the washing process. No damage to the RPE layer integrity is noted.
Figure 3.7: Histological sections of RPE eyecup immunostained with anti-MHC class I antibodies

Posterior eyecups were produced from enucleated eyes of adult C57BL/6 mice and stained with FITC-conjugated anti-MHC class I antibody and viewed with confocal microscope

A. Untreated posterior eyecup showing low level of MHC class I

B. Posterior eyecup treated with IFN-g for 12 hours prior to staining showing upregulation of MHC class I.
Figure 3.8: Confocal appearance of RPE layers from eyes of C57BL/6 and B6.gld mice after treatment with IFN-γ and incubation with effector T cells

A. RPE eyecup treated with IFN-γ for 12 hours without the addition of BALB/c effector T cells. Addition of IFN-γ does not induce toxicity in the RPE layer on its own.

B. RPE eyecup treated with IFN-γ for 4 hours prior to exposure to pre-sensitised splenocytes. A small number of RPE cells have died as evidenced by co-staining of the RPE cell nuclei with both AO and PI.

C. RPE eyecup treated with IFN-γ for 12 hours prior to incubation with pre-sensitised splenocytes. A larger number of RPE cells display yellow staining nuclei (combination staining with AO and PI) indicating greater death.
Figure 3.8
3.3 Discussion

In age-related macular degeneration early changes in and ultimately loss of RPE is thought to be central to the pathogenesis (Porto 2002). In some subset of Leber’s amaurosis, dysfunction and loss of RPE is thought to be the primary pathology in which mutation of a protein uniquely expressed in RPE renders these cells incapable of supporting photoreceptors, and eventually the retina deteriorates (Yao 1992). For these and similar diseases, transplantation of allogeneic RPE to replace defective or depleted RPE is an attractive potential clinical solution.

Allogeneic tissues as grafts are vulnerable to immune rejection and this potential exists in principle for allogeneic RPE grafts. The importance of this potential is mitigated, on the one hand, because the subretinal space in which RPE grafts are to be placed is an immune privileged site. Additionally there is unequivocal evidence that RPE tissue has properties of an immune privileged tissue (Wenkel 2000). On the other hand RPE express transplantation antigens that should make them vulnerable to immune recognition (Zhang 1998) and there is evidence that allografts of RPE placed intraocularly, even in the subretinal space, are subject to destruction that is presumed to be immunological in nature (Streilein 2002).

The results above, indicate that explanted, intact layers of RPE resident in posterior eyecups are quite resistant to lysis by Tc that are fully able to lyse other types of genetically identical target cells. Moreover, this resistance to lysis is unrelated to expression of CD95L because Tc layered onto RPE of posterior eyecups prepared from CD95L-deficient mice was also unable to cause lysis. The lack of protection by CD95L is surprising since CD95L expression on corneal endothelium has been linked causally to the acceptance of orthotopic corneal allografts in mice (Yamagami 1997). Similarly, our laboratory has demonstrated that CD95L expression on allogeneic neonatal RPE grafts placed heterotopically (beneath the kidney capsule) protects the grafts from immune rejection (Wenkel 2000).

Sub-threshold expression of the MHC class I molecules may help to explain the resistance of explanted RPE to Tc-mediated lysis. When posterior eyecups were treated for 12 h with IFN-γ, class I expression on the RPE was enhanced, and specific Tc were able to kill a modest, but significant, proportion of RPE treated in this manner. Low level constitutive expression of the MHC class I molecules is a characteristic feature of ocular cells in addition to RPE. Corneal endothelial cells are especially depauperate in class I expression and this is
believed to explain why donor-specific cytotoxic T cells induced in mice bearing the MHC-disparate orthotopic corneal allografts play no role in graft rejection (Yamada 2001). Moreover, one doubts that low level class I expression alone is sufficient to account for the resistance of RPE to Tc-mediated lysis. RPE is known to secrete soluble factors that suppress T cell activation, and these factors may also protect RPE from T effector cell attack.

The resistance to Tc-mediated lysis displayed \textit{in vitro} by RPE in posterior eyecups was mirrored to some extent when specifically sensitised T cells were injected into the subretinal space of appropriate recipients. The positive result is that effector T cells created small, circumscribed lesions at the site of injection, and eventually these sites were found to be depleted of RPE. The surprise in this result is that these lesions failed to progress and expand through time. When Tc are added to non-ocular target cells \textit{in vitro}, they are able through time to eliminate every target cell. Yet, Tc placed in the subretinal space failed to display this capacity. Moreover, the constraint applied to the injected cells was not provided by CD95L. Lesions produced in the RPE layer of B6.\textit{gld} eyes were similar in all respects to those produced in wild type C57BL/6 eyes. It should be pointed out that the lymphoid cell suspensions injected into the subretinal space in these experiments contained allo-specific CD4\textsuperscript{+} T cells, as well as Tc. This is important because histological examination of injected eyes revealed almost no non-specific inflammation at the injection site. We interpret this to mean that RPE themselves secrete, or the subretinal space in which the cellular inocula were placed contains, factors that silence the destructive potential of effector T cells. Determining the nature and mode of action of these silencing factors is a major goal of the subsequent chapters.
Chapter 4 – RPE inhibits T cell activation by releasing soluble factors

4.1 Introduction

Retinal transplantation has gained momentum recently with the advent of new techniques for growing RPE cells as intact monolayers, and the success of neural stem cell transplants in providing integration between the host retina and grafted materials (Tezel 1999, von Recum 1998, Young 2000). Grafts have been placed in the subretinal space of normal eyes, as well as of eyes with various degenerative diseases or after local trauma. The vulnerability of foreign tissue grafts in the subretinal space is likely to be influenced by the immune status of the space – normal or deranged by disease or experimental manipulation. Understanding the parameters that dictate graft vulnerability is an important goal if successful retinal transplantation is to be a therapeutic hope for the future.

The subretinal space under normal circumstances contains virtually no free fluid. However, during pathological conditions such as spontaneous rhegmatogenous retinal detachments this potential space expands and fills with fluid. Anatomically the subretinal space is isolated from the cells and molecules of the immune system, on the one hand, by the presence of the outer blood retinal barrier (BRB) formed by the tight junctions between the apico-lateral surfaces of retinal pigment epithelial cells (RPE) cells, and, on the other hand, but the interlocking foot processes of Müller cells that surround retinal photoreceptor cells forming the outer limiting membrane. The vessels of the neural retina also possess a formidable blood: tissue barrier. The interphotoreceptor matrix of the subretinal space is largely formed by the RPE.

In part because of its anatomic sequestration, the subretinal space has been demonstrated to be an immune privileged site. Jiang and his co-workers showed that neonatal retinal allografts as well as neonatal RPE allografts from C57BL/6 mice transplanted into the subretinal space of the MHC and minor antigen incompatible BALB/c recipients survived for extended period of time compared to similar grafts placed subcutaneously (Jiang 1991). Additionally, Wenkel et al. showed that allogeneic P815 tumours placed in the subretinal space of BALB/c mice survived for a prolonged period of time as compared to their survival
subcutaneously (Wenkel 1998). Mice bearing allogeneic P815 tumour grafts in their subretinal space also failed to acquire P815-specific delayed hypersensitivity (DH). Therefore, the subretinal space displays two important features of an immune privileged site: it accepts for prolonged intervals grafts of allogeneic tissues, and it promotes the induction of a systemic immune deviation to antigenic material placed within the site.

The aim of these sets of experiments was to detect the contribution of RPE to the immune privileged status of the subretinal space. Soluble factors, specifically Transforming growth factor β (TGF-β), somatostatin (SOM) and thrombospondin-1 (TSP-1), have been shown to have a role in diminishing inflammatory responses. In the following segment TGF-β, TSP-1 and SOM will be described in greater details and the evidence for their anti inflammatory functions will be elucidated. Since the putative main aim of immune privilege is to diminish inflammatory responses and since interferon-γ (IFN-γ) is the hallmark of inflammation produced by Th1 cells, this cytokine will also be briefly described.

4.2 Transforming growth factor-β: An overview

Transforming growth factor-β (TGF-β) is comprised of a family of structurally related 25-kilodalton disulfide-linked dimeric proteins secreted by nearly all cell types. TGF-β is secreted as a biologically inactive complex consisting of latent TGF-β and the latent TGF-β binding protein (LTBP). Latent TGF-β is composed of mature TGF-β associated noncovalently to its amino-terminal propeptides, the latency associated peptide (LAP). LTBP is a 120-190 kDa glycoprotein that is disulfide-linked to LAP, and has been demonstrated to increase secretion of latent TGF-β, as well as participating in its activation (Nunes 1995, Flaumenhaft 1993, Miyazono 1991).

There are three known isoforms of TGF-β, TGF-β1, β2 and β3 all of which are produced in latent form and are 72-79% similar to each other in primary amino acid sequence (Lawrence 2001). The multiple activities of TGF-β can be divided into three main functions: Inhibition of cell proliferation; increasing the extracellular matrix deposition, particularly fibrin deposition; and complex immunoregulatory properties (Lawrence 1996). For all three isoforms of TGF-β to exert their diverse effects, activation of the latent TGF-β complex is required.
4.2.1 TGF-β activation

Latent TGF-β can be converted into its active form by physiochemical agents such as treatment with heat/pH extremes, addition of plasmin or cathepsin D, addition of chaotropic agents, or cleavage of carbohydrate moieties of latent TGF-β (Nunes 1995, Lyons 1990, Lyons 1988, Brown 1990). In addition, a variety of tissue culture systems such as co-culture of vascular endothelial and smooth muscle cells have been described in which endogenously secreted latent TGF-β is activated. The mechanisms of activation of TGF-β in vivo are not fully elucidated. Since there are multiple ways of activating TGF-β in vitro, it may be suggested that several activation mechanisms also exist in vivo and depending on the cell type and the function required from TGF-β molecule, a particular mechanism may be the dominant or the exclusive method. Acidification may also be a mechanism of activation of TGF-β in vivo (for example in the acidic environment of the activated macrophages) (Silver 1988). Low pH may also activate secreted lysosomal proteases, which may lead to subsequent TGF-β activation (Montcourrier 1997).

Ionising radiation was found to activate TGF-β in vivo, but mesangial cell cultures radiated in vitro did not exhibit activated TGF-β (Barcellos-Hoff 1993, O’Malley 1999). Pharmacological agents such as vitamin D3, via reduction of plasminogen activator (Koli 1996); dexomethasone via increase secretion of lysosomal proteases (Grainger 1995); aspirin (Grainger 1995) and IL-1β (Studer 1999) are able to activate TGF-β indirectly. Retinoids (Vitamin A derivatives) up-regulate plasminogen activator and hence plasmin activity leading to formation of active TGF-β (Kojima 1993). However plasminogen knock out animals do not exhibit the same pathology as TGF-β1 knock out mice, and therefore plasmin is only one of the ways by which is TGF-β is activated (Shull 1992).

Thrombospondin has been shown to be an important activator of TGF-β both in vitro and in vivo. Thrombospondin and its role in activation of TGF-β is discussed in subsequent segments.

4.2.2 Functions of TGF-β

Almost all cells in the body produce TGF-β and have one or more of its receptors. TGF-β participates in proliferation and differentiation of cells, wound healing, angiogenesis and embryonic development. All three isoforms share these functions but depending on the
type and state of the cell, "exhibit temporal and spatial differences in their expression" (Lawrence 2001). TGF-β₁ is present in endothelial, connective tissue and haematopoietic cells, TGF-β₂ in epithelial and neuronal cells and TGF-β₃ in mesenchymal cells (Massague 1998). TGF-β is important in cancer susceptibility, development and progression. Due to its anti-proliferative function, TGF-β is a potent inhibitor of stromal, epithelial and haematopoietic cell growth. However at some point in the development of cancers, the cells become resistant to TGF-β action by losing TGF-β receptors on their surface or due to inactivating mutation, or loss of expression of the genes for the components of the TGF-β signalling pathway (Pasche 2001). Later still, tumours actively secrete TGF-β, which contributes to angiogenesis, metastasis and decreased host immune response to the tumour (Pasche 2001), leading to tumour progression.

4.2.3 Interactions of TGF-β with various parts of the eye

All three isoforms of TGF-β are formed in the eye with TGF-β₂ predominating. In the anterior segment of the human eye, TGF-β₁ is found in superficial limbal epithelial cells as well as in the stroma proximal to ciliary processes. TGF-β₂ in addition has been found in conjunctival epithelium as well as in the epithelium adjacent to pars plana (Pasquale 1993). Only TGF-β₂ has been found in aqueous humour (Jampel 1990). Pfeffer et al. also found that TGF-β₂ is the predominant isoforms found in the neural retina, RPE-choroid complex and vitreous of monkey eye (Pfeffer 1994). TGF-β₃ is found in isolated cells in the choroid and retina (Lutty 1993) and anti TGF-β₃ antibodies were noted next to the apical aspect of RPE (Anderson 1995).

TGF-β is implicated in some ocular pathology including glaucoma, PVR and cataract formation (Tripathi 1994, Picht 2001, Limb 1991, Pena 1994, Hales 1995, Lovicu 2002). TGF-β levels in the aqueous humour of glaucomatous eyes are significantly higher that the matched controls (Li 1996) and the levels may correlate with progression of POAG and diabetes (Ochiai 2002). There are increased expression of TGF-β₁ and TGF-β₂ in glial cells around the lamina cribrosa of monkey’s optic nerve (Fukuchi 2002). Activated microglia secreting abundant TGF-β₂ were found around the glaucomatous optic nerve head of human as compared to normal matched controls (Yuan 2001).
TGF-β induces aberrant growth and differentiation of lens epithelial cells in rodents mostly through TGF-β induced apoptosis. Similar molecular and morphological features lead to formation of anterior subcapsular and posterior capsular opacification in human (Maruno 2002). There is evidence to suggest that the susceptibility to TGF-β₂ induced cataract increases as rats age (Hales 2000). There are increased levels of TGF-β₁, LTBP₁ and TGF-β₂ in lens fibrils in pseudoexfoliation syndrome (Schlotzer-Scherhardt 2001).

All three isoforms of TGF-β enhanced scar formation after conjunctival surgery and TGF-β₂ even counteracted the anti scarring effect of anti metabolite, mitomycin C (Cordeiro 1999). TGF-β seems to also be important in formation and progression of proliferative vitreoretinopathy (PVR) (Oshima 2002). TGF-β₁ induces the expression of α-smooth muscle actin from RPE cells and significantly increased the synthesis of fibronectin by RPE cells which may lead to fibrin deposition and development of PVR (Stocks 2001). Retinal detachment induces synthesis and secretion of TGF-β₂ in vitreous, probably by Müller cells.

TGF-β₂ has an inhibitory effect on endothelial cell growth and hence angiogenesis in a dose dependent manner (Hayasaka 1998). Pan retinal photocoagulation (PRP) that is used in diabetic retinopathy to inhibit new vessel growth, up-regulates mRNA expression of both TGF-β₁ and TGF-β₂. Additionally concentration of TGF-β₂ rises in the vitreous but not the aqueous humour (Matsumoto 1994, Ishida 1998). This up-regulation of TGF-β may be responsible for inhibition of endothelial cell growth, and hence new vessel formation, which is a consequence of pan retinal photocoagulation (PRP) (Yamamoto 1998).

TGF-β₂ inhibits growth and DNA synthesis of cultured uveal melanocytes (Hu 1998). In glial cells, TGF-β increases the mRNA expression and protein secretion of tissue plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI)-1 (Schacke 2002).

4.2.4 Role of TGF-β as an immunomodulator

TGF-β performs a complex balancing act as far as controlling the immune system is concerned. Early in the immune response, the released TGF-β from platelet stores acts as chemo attractant for monocytes, macrophages, fibroblasts and neutrophils (Ling 2002, Gruber 1994). T cell activated in the presence of TGF-β show an increase in proliferation and IL-2 production (Cerwenka 1994), while addition of anti-TGF-β antibody to LPS-activated cultures decreased the levels of IgG1-3 and IgE significantly (Snapper 1993). Co-injection of
recombinant TGF-β with LPS into the vitreous of rabbits reduced inflammatory cell infiltration leading to reduction of uveitis as compared with injection of LPS alone (Allen 1996). However later in the immune response, TGF-β inhibits the functions of activated T and promotes apoptosis in order to down regulate the inflammatory process. TGF-β inhibits proliferation and cytokine production by both Th1 and Th2 and hence endogenous TGF-β is protective against both Th1 cell-mediated autoimmunity and allergic inflammation. TGF-β has an ability to attract macrophages, which in turn ingest apoptotic cells and reduce pro-inflammatory signals contributes to limiting the inflammation.

4.3 Somatostatin: An overview of structure and function

Somatostatin (SOM) is a tetra-deca-neuro-peptide with wide distribution in the CNS, CSF, pancreas and gut and a diverse function as a neurotransmitter, anti-secretory and anti-proliferative agent (Patel 1999). The biological effects of SOM are mediated via 5 cell surface receptors (SSTR1-5). SOM inhibits in vitro secretion of GH and slows synthesis and release of thyroid stimulating hormone (TSH) and prolactin. Elsewhere it inhibits basal secretion of insulin and glucagon from the pancreas and gastrin and secretin from the gut mucosa. SOM has been shown in several in vitro and in vivo systems to limit inflammation: Injection of SOM into joints of patients with rheumatoid arthritis has shown a reduction in synovitis (Coari 1995) and usage of a synthetic agonist of SOM in mice infected by Schistosoma Mansoni diminishes the size of the hepatic granulomas elicited by eggs of the parasite (Blum 1992). Moreover SOM was shown to inhibit IFN-γ production by Ag-stimulated granuloma cells and splenocytes of schistosome-infected mice (Blum 1992) and diminish mitogen-induced activation of lamina propria mononuclear cells and of the “autologous peripheral blood lymphocytes” (Fais 1990). SOM also inhibited proliferation of human peripheral blood lymphocytes (Atiya 1997). Granuloma macrophages were shown to have mRNA for SOM (Elliott 1998) and secret the protein (Weinstock 1990) for SOM. SOM was shown to be stimulated by dexomethasone in carrageenin-induced inflammation in SD rats and thus may have a participatory role in anti-inflammatory actions of glucocorticoids (Karalis K 1995). SOM has been used in place of corticosteroid in the treatment of Grave’s Ophthalmology and shown to have comparable efficacy.

Somatostatin has been found in ganglion cell layer, inner nuclear (INL) and inner plexiform (IPL) and nerve fibre (NFL) layers of retina (Schindler 1996, Mitrofanis 1989, Li
1990, Kossut 1989) and various SOM receptors have been found in the outer plexiform layer (OPL), INL, RPE, iris, ciliary body and the choroid in human (Lambooij 2000, Klisovic 2001).

4.4 Thrombospondin: A brief description of structure and function

Thrombospondins are a family of multimeric, multidomain glycoproteins that function at cell surface or as a part of the extracellular matrix (Adams 2001) and participate in communications between cells and cells and matrix (Lawler 2000). TSP-1 is readily purified from platelets and is, therefore, the most commonly studied member of the family. Both TSP-1 and TSP-2 inhibit angiogenesis in both in vivo and in vitro assays (Tolsma 1993). Moreover, over-expression of TSP-1 or -2 in tumour cells leads to inhibition of angiogenesis and tumour growth (Bleuel 1999, Streit 1999). Peptides derived from TSP-1 were shown to inhibit endothelial cell growth in a retinal explant assay (Shafiee 2000). The anti angiogenic activity of TSP has been attributed by various investigators to its ability to activate latent TGF-β (Schultz-Cherry 1995), to bind to CD36 (Dawson 1997) or to interact with heparan sulfate proteoglycans (Guo 1992).

Perhaps one of the most important functions of TSP is its ability to activate TGF-β. TSP-1 binds to small latent TGF-β complex (TGF-β-LAP) leading to the release of mature active TGF-β independent of the presence of proteases (Murphy-Ullrich 2000) and is known to be a major activator of TGF-β both in vitro and in vivo (Murphy-Ullrich 2000, Crawford 1998). The evidence for the role of TSP in TGF-β activation in vivo comes mainly from TSP-1 null mice: TSP-1 null mice have an altered dermal wound healing response associated with a decrease in TGF-β activity extracted from the wound and characterised by prolonged capillary angiogenesis and macrophage infiltration (Murphy-Ullrich 2000). The abnormalities noted in the lungs of TSP-1 deficient mice are similar to those found in the lungs of TGF-β-deficient mice (Crawford 1998).

Because of the small size of the mouse eye, it is not technically possible to gain access to the subretinal space without undue trauma - which runs the risk of altering the native status of immune privilege. Moreover, insufficient amounts of interphotoreceptor matrix exist to harvest for in vitro analysis. Consequently, the posterior eyecup model was produced and
incubated with serum free medium. The supernatant (SN) of eyecups from eyes of C57BL/6 and thrombospondin-1 knock-out (TSP-1KO) mice were used to study the interaction of soluble factors produced by RPE and activated T cells.

4.5 Results

4.5.1 Effect of supernatants of RPE eyecups on T cell proliferation

Supernatants from cultured monodisperse RPE cells have been shown to inhibit T cell proliferation, an expression of T cell activation, by elaborating a soluble factor (Farrokh-Siar 1999). To see if the intact monolayer of RPE in RPE eyecups is also capable of secreting factors that inhibit T cell proliferation, SN of RPE eyecups was collected after the preparations were cultured with SFM for 24 hours. 4 x 10^5 DO11.10 lymph node cells were incubated with diluted RPE SN and ovalbumin (OVA) for 96 hours at 37°C. ^H-thymidine was added to each well on day 4 and incubated over night, and uptake was measured. As a positive control, some wells contained DO11.10 lymph node cells with OVA in the absence of RPE SN. Wells with only DO11.10 lymph node cells and SFM served as negative controls. Figure 4.1A shows that DO11.10 T cells are capable of mounting strong proliferative responses to OVA, and that RPE supernatants inhibited this proliferation in a concentration dependent manner. Even at dilution of 1:40, the anti proliferative capacity of RPE SN was significant. Thus, RPE monolayers cultured as posterior eyecups secrete soluble factors into the medium that inhibit T cell proliferation.

4.5.2 Effect of supernatants of RPE eyecups on IFN-γ production by T cells

Proliferation is only one parameter that expresses the state of T cell activation. Since AQH has been shown to deviate precursor T helper cells away from both Th1 and Th2 phenotypes (Taylor 1997), it was worth assessing whether the SN harvested from RPE eyecups would behave similarly. Moreover, ocular immune privilege has been found to suppress T cells that secrete Th1 type cytokines, such as IFN-γ. The following experiments examined this point. DO11.10 lymph node cells were incubated with OVA and diluted SN of RPE eyecups. The SN were removed from these cultures at 96 hours and examined for content of IFN-γ by ELISA. As a positive control, DO11.10 lymph node cells were incubated with OVA alone to demonstrate maximal IFN-γ production. DO11.10 lymph node cells incubated with only SFM served as negative control. The results displayed in Figure 4.1B
reveal that DO11.10 T cells are capable of producing appreciable amounts of IFN-γ in response to OVA, and that IFN-γ production is significantly reduced when the T cells are stimulated in the presence of SN of RPE eyecups. Thus, SN from RPE eyecups contains a soluble factor(s) that suppresses T cell activation in vitro by two parameters.
24 hours after incubation of RPE eyecups with SFM, SN was collected. DO11.10 lymph node cells were incubated with OVA and serially diluted SN of RPE for 96 hours at 37°C. Positive control represents wells containing DO11.10 lymph node cells with OVA and negative control denotes wells containing only DO11.10 lymph node cells and SFM.

A. $^3$H-thymidine was added to some wells on day 4 and incubated over night. Thymidine uptake was measured as a function of T cell proliferation.

B. Supernatants of the other wells were collected at 96 hours and used in IFN-γ ELISA. Bars represent the mean ± SEM of triplicate wells from a representative experiment. * and ** indicate mean values significantly greater than the positive control effect ($p<0.05$ and $p<0.01$) respectively.
4.5.3 Role TGF-β mediates in T cell suppression by RPE eyecup supernatants

RPE cells constitutively contain mRNA for TGF-β₁, TGF-β₂ and TGF-β₃ (Tanihara, 1993), and TGF-β₂ was found to be the predominant isotype in the retina and RPE, as well as in the aqueous humour of monkeys (Pfeffer 1994). TGF-β₁, mostly in the latent form, was the first immunosuppressive factor identified in normal AqH. When activated, TGF-β₂ profoundly inhibits both proliferation and lymphokine production by T cells (Cousins 1991). The first impulse was to determine whether TGF-β is present in RPE eyecup supernatants, and if so, whether it was responsible for the capacity of these SN to suppress T cell activation. To identify the presence of TGF-β, the mink lung epithelial cell proliferation inhibition assay was used. Supernatants were harvested from RPE eyecups that were incubated in SFM for 0, 1, 6, 12, 24 and 48 hours, diluted, divided in halves so that one portion could be acid treated (to reveal the total amount of TGF-β present), while the other portion was assayed directly without acid treatment (to reveal the amount of active TGF-β present in the SN). Mink lung cells were incubated with the treated and untreated SN for 20 hours, then ³H-thymidine was added, and the amount of isotope incorporated assessed. In order to determine the isotype of TGFβ present in the SNs, some samples were treated with anti-TGFβ₁ and/or anti-TGFβ₂ prior to addition to the mink lung cells. The results of these experiments are presented in Figure 4.2. Within one hour of incubation of RPE eyecups in SFM, latent (but not active) TGF-β was detected. The concentration of total TGF-β in subsequent supernatants increased to a maximum at 12 hours post incubation (Figure 4.2A). Active TGF-β was first detected at 6 hours of incubation, and peak active TGF-β levels were reached at 24 hours after incubation. Plateau levels of total TGF-β began to decline after 48 h of incubation (Figure 4.2A). In contrast to the SN incubated with anti-TGF-β₁, the SN mixed with anti-TGF-β₂ antibodies alone was almost completely inhibited, implying that TGF-β₂ is virtually the exclusive isoforms secreted by the apical surface of RPE cells in RPE eyecups. These results are interpreted to mean that RPE eyecups elaborate latent TGF-β, which they then convert into the active form by a mechanism yet to be revealed.
Figure 4.2: Kinetics of TGF-β production and activation in SN of RPE eyecups and effect of anti-TGF-β antibody and soluble TGF-β receptor III on DO11.10 T cell proliferation in the presence of RPE SN

A. Posterior eyecups were incubated with SFM for 1, 6, 12, 18, 24 and 48 h. After these time intervals the SN was collected and diluted. Mv1Lu cells from sub-confluent cultures were incubated with unmodified or acid-treated SN of the RPE eyecups. After incubation for 2, 3H-thymidine was added and 4 hours later the amount of incorporated label was measured.

B. Eyecups from C57BL/6 mice were incubated in SFM for 24 hours and their SN collected and diluted 1 in 10. DO11.10 lymph node cells were incubated with SN of RPE and OVA. 3H-thymidine was added to each well on day 4 and incubated overnight. Thymidine uptake was measured as a function of T cell proliferation. Positive control wells contained lymph node cells and OVA and negative control wells contained only lymph node cells in SFM. Experimental wells contained untreated RPE SN, SN treated first with anti-pan-TGF-β antibody for an hour or SN treated first with soluble TGF-β receptor III.

Bars represent the mean ± SEM of triplicate wells from a representative experiment.

** indicates mean values significantly greater than the positive control effect (p<0.01).
Figure 4.2

A

Eyecup incubation prior to SN collection

- Latent TGF-β
- Active TGF-β

B

Positive Control
Negative Control
C57BL/6 SN
In order to determine whether the active TGF-β present in RPE eyecup SN was responsible for inhibiting T cell activation in vitro, a pan anti-TGF-β antibody reagent was next added to 24-hour RPE eyecup SN prior to suspending DO11.10 lymph node cells in the medium in the presence of OVA. As controls, DO11.10 lymph node cells were incubated with OVA alone, with SFM alone or with OVA and untreated SN of RPE eyecup. Figure 4.2B demonstrates that neutralisation of active TGF-β by the antibody reversed the inhibitory effect of 24-hour RPE SN on DO11.10 T cells proliferation, but only partially. In case the anti-TGF-β antibody failed to neutralise all TGF-β, an additional experiment was performed in which soluble TGF-β-RIII was also added to SN containing anti-TGF-β antibodies. As revealed in Figure 4.2B, the combination of anti-TGF-β antibodies and soluble TGF-β-RIII further relieved the suppression inherent in the RPE SN – but still not completely. These results confirm that TGF-β contributes to the ability of RPE eyecup SN to suppress T cell activation, but the results also suggest that other immunosuppressive factors are also present.

4.5.4 Relationship between T cell suppression and presence of active TGF-β

In order to ascertain what role the activation of TGF-β plays in T cell inhibition by RPE SN, supernatants were removed at 0, 1, 6 and 24 h after incubation with SFM. These SN were diluted and placed in T cell proliferation and IFN-γ production assays as previously described. As revealed in Figure 4.3, SN of RPE eyecups harvested after 0 and 1 hour incubation failed to inhibit T cell proliferation. Only after 6 hours and thereafter did SN suppress T cell proliferation, and these time points correspond to the presence of active TGF-β. IFN-γ production was also inhibited at 6 hours incubation and beyond, but it was also reduced at 1 h compared to positive control. These results support the view that activation of TGF-β in RPE SN is necessary for T cell proliferation to be suppressed. However, the results also imply that either another immunosuppressive agent(s) is present to inhibit IFN-γ production, or that latent TGF-β can achieve this result.
Figure 4.3: Correlation between the duration of RPE eyecup incubation and its ability to suppress T cell activation

RPE eyecups were incubated with serum free media for 1, 6, 24 and 48 hours, and then SN was collected and diluted. DO11.10 lymph node cells were incubated with OVA and different SN. Positive control represents wells containing DO11.10 lymph node cells with OVA. Negative control denotes wells containing only DO11.10 lymph node cells and SFM.

A. $^3$H-thymidine was added to each well on day 4 and incubated over night. Thymidine uptake was measured as a function of T cell proliferation.

B. Supernatants of the wells were collected at 96 hours and used in IFN-γ ELISA. Bars represent the mean ± SEM of triplicate wells from a representative experiment.

* and ** indicate mean values significantly greater than the positive control effect ($p<0.05$ and $p<0.01$) respectively.
Figure 4.3

A

![Graph A](image)

**Hours of eyecup incubation before SN collection**

B

![Graph B](image)

**Hours of eyecup incubation before SN collection**

- **Positive Control**
- **Negative Control**
- **C57BL/6 SN**
Figure 4.4: Expression of mRNA for TSP and SOM in RPE cells by RT-PCR

Total RNA extracted from freshly isolated and cultured RPE cells was subjected to RT-PCR analysis using pairs of specific primers for Thrombospondin (TSP), preprosomatostatin (ppSOM) and housekeeping gene GAPDH, using conditions described in Methods and Materials. PCR products were analysed by ethidium bromide/agarose gel electrophoresis.
Figure 4.4

RPE derived from

Cultures  Eye cups  Neg.cont.  (100 bp)

ppSomatostatin  500 bp
(265 bp)

Thrombospondin  500 bp
(733 bp)

GAPDH  500 bp
(245 bp)
4.5.5 Role of thrombospondin-1 (TSP-1) in activation of TGF-β in eyecup SN

A variety of factors and conditions have been demonstrated to be capable of activating TGF-β from its latent form. Particular attention was paid to thrombospondin because cultured RPE cells express mRNA for TSP-1, 2, 3 and 4 by RT-PCR (Carron 2000), and cultured human RPE cells have been shown to secrete TSP-1 (Miyajima-Uchida 2000). TSP-1 has recently been found to play a central role in conferring immunoregulatory properties on conventional APCs and thus directly alters the functional properties of Th1 cells otherwise destined to become Th1 like (Masli 2002).

To demonstrate that RPE eyecups can produce TSP, PCR for TSP-1 was performed. Figure 4.4 demonstrates that posterior eyecups of C57BL/6 mice contained easily detectable TSP mRNA transcripts. In order to determine if TSP plays an important role in activating TGF-β found in RPE eyecup SN, eyecups were prepared from eyes of TSP-1 knock out mice (TSP-1KO). SN harvested from these eyecups after 24 h incubation were diluted 1:10, and then divided in half. One portion was treated with HCl to activate all the TGF-β present (for assessment of total TGF-β); the other portion was not treated (for assessment of active TGF-β). The supernatants were then assayed for the presence of active and total TGF-β using the mink lung cells. The results presented in Figure 4.5 indicate that SN of RPE eyecups of TSP-1KO mice contained comparable amounts of total TGF-β to that found in SN of normal RPE eyecups. However, virtually no active TGF-β was found in TSP-1KO SN.

To reveal the functional importance of TSP-1-dependent TGF-β activation in these studies, TSP-1KO RPE SN were tested for their capacity to suppress activation of DO11.10 T cells in vitro, as described previously. Figure 4.6 demonstrates that, as before, RPE eyecup SN from normal C57BL/6 mice suppresses T cell proliferation and IFN-γ production. SN from TSP-1KO eyecups was significantly less able to suppress proliferation and IFN-γ production than SN from normal RPE eyecups. However, as compared to the positive control, the SN from TSP-1KO eyecups was still able partially to inhibit both proliferation and IFN-γ production by DO11.10 T cells. These results point strongly, as do the results described above, to the presence of additional immunosuppressive factors in supernatants of RPE eyecups.

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Figure 4.5: Total and active TGF-β content of SN of eyecups prepared from C57BL/6 mice and TSP KO mice

Posterior eyecups were prepared from C57BL/6 and TSP-1KO mice, and SN were collected after 24 hours of incubation in SFM. Mv1Lu cells from sub-confluent cultures were incubated with unmodified or acid treated SN. $^3$H-thymidine was added to each well and 4 hours later the amount of incorporated label was measured. Bars represent the mean ± SEM of triplicate wells from a representative experiment. ** indicate mean values significantly greater than C57BL/6 SN effect ($p<0.01$).
Figure 4.5

![Graph showing TGF-β levels in TSP KO and C57BL/6 mice.](image)

- Active TGF-β
- Total TGF-β
Figure 4.6: Effects of SN of C57BL/6 and TSP-1KO RPE eyecups on proliferation and IFN-γ production by DO11.10 T cells

SN of RPE eyecups from C57BL/6 and TSP-1KO mice were removed 24 hours after incubation with SFM. DO11.10 lymph node cells were then co-cultured with OVA and diluted SN. Positive control wells contained lymph node cells and OVA and the negative control wells only lymph node cells in SFM.

A. ³H-thymidine was added to each well on day 4 and incubated over night. Thymidine uptake was measured as a function of T cell proliferation.

B. On day 4, the supernatants of the wells were collected and used in IFN-γ ELISA. Bars represent the mean ± SEM of triplicate wells from a representative experiment.

** indicate mean values significantly greater than C57BL/6 SN effect (p<0.01).
Figure 4.6

A

![Chart A showing CPM values for Positive Control, Negative Control, TSPKO SN, and C57BL/6 SN.](chart-a)

B

![Chart B showing IFN-γ (ng/ml) values for Positive Control, Negative Control, TSPKO SN, and C57BL/6 SN.](chart-b)
4.5.6 Role of somatostatin in T cell suppression by RPE eyecup SN

Somatostatin (SOM) was chosen as a candidate for several reasons: SOM inhibits IFN-γ production by T cells in murine schistosomiasis and by human peripheral blood monocyte cells (Muscettola 1990, Blum 1992). SOM inhibits production of IL-2R positive cells after mitogen stimulation of mononuclear cells (Fais 1991). Taylor et al. have reported that SOM is present in AqH and that it suppresses IFN-γ production by activated T cells (Taylor 2003). Finally, transcripts for SOM have been reported to be present within the retina (Sagar 1985). In order to ascertain the production of SOM by RPE in posterior eyecups, both RT-PCR and ELISA assays were performed.

Figure 4.4 shows that RPE cells both in monodisperse culture and harvested from eyecups express mRNA for somatostatin. Moreover, SN of RPE eyecups contain large amounts of SOM in a dilution dependent manner (see Figure 4.7). In order to determine whether SOM participates in inhibition of T cell activation, SN of RPE eyecups from C57BL/6 mice were treated with antibody to SOM and used in DO11.10 T cell proliferation and IFN-γ production assays. As revealed in Figure 4.8, SN with SOM blocked by anti-SOM antibody was much less efficient at suppressing T cell proliferation and IFN-γ production. However, neutralising SOM did not completely eliminate the SN capacity for T cell suppression. In fact, simultaneous neutralisation of both SOM and TGF-β with appropriate antibodies, largely, but incompletely abolished SN inhibitory activity. It can be concluded that (a) SOM is produced by RPE monolayers in eyecups and SOM accounts for some of the T cell inhibitory capacity of these supernatants; and (b) that SOM and TGF-β together are the major factors produced by RPE monolayers to create an immunosuppressive microenvironment. The evidence does not suggest that these two factors act synergistically.
Figure 4.7: SOM production by RPE

RPE eyecups prepared from eyes of C57BL/6 mice were incubated with SFM for 24 hours, SN were removed and diluted 1:10, 1:20 and 1:40 prior to being tested for SOM content by ELISA. Bars represent the mean ± SEM of triplicate wells from a representative experiment.
Figure 4.7

Dilution of SN of RPE

SOM (ng/ml)
Figure 4.8: Effect of inhibition of SOM in RPE eyecup SN on T cell activation.

SN of RPE eyecups from C57BL/6 mice were removed 24 hours after incubation with SFM and placed together with DO11.10 lymph node cells and OVA in T cell activation assays. Positive control wells contained lymph node cells and OVA and the negative control wells only lymph node cells in SFM.

A. $^3$H-thymidine was added to each well on day 4 and incubated over night. Thymidine uptake was measured as a function of T cell proliferation.

B. On day 4, the supernatants of the wells were collected and used in IFN-γ ELISA. Bars represent the mean ± SEM of triplicate wells from a representative experiment.

* and ** indicate mean values significantly greater than the C57BL/6 SN effect ($p<0.05$ and $p<0.01$) respectively.
Figure 4.8

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4.5.7 Effect of SN of RPE eyecup on activation of T cells from TGF-β receptor II dominant negative mice

Since both antigen presenting cells (APC) and T cells are present in the same wells in the DO11.10 lymph node assay, we can not be certain if the effects of RPE SN on T cell activation was due to a direct effect on T cells or due to an effect on APCs. Additionally, the role of TGF-β present in the SN of RPE in T cell inhibition was confirmed by using T cells from TGF-β RII DN mice that do not respond to TGF-β. Lymph nodes and spleens were harvested from naïve C57BL/6 and TGF-β RII DN mice. Purified T cells were co-cultured with anti-CD3 antibody and 24 h SN of eyecups from eyes of C57BL/6 mice, after which, T cell proliferation was measured. Some cultures were allowed to proceed for 48 hours and their supernatants were collected for IFN-γ measurement. Wells with only T cells and anti-CD3 antibody served as positive controls and those containing only T cells in SFM served as negative controls. Our results are depicted in Figure 4.9. T cells from both WT and TGF-β RII DN mounted comparable proliferative responses to anti-CD3 antibody, and they produced similar levels of IFN-γ. Whereas SN of RPE significantly inhibited both proliferation and IFN-γ production by WT C57BL/6 T cells, the SN failed to inhibit proliferation of TGF-β RII DN T cells. By contrast RPE SN readily inhibited IFN-γ production by anti-CD3 stimulated TGF-β RII DN T cells. It can be concluded that (a) the inhibitory effect of RPE SN is aimed at T cells, and (b) TGF-β is the major inhibitor of T cell proliferation. The inference can be drawn that there are other factors in SN, that are responsible for inhibiting IFN-γ secretion by anti-CD3 stimulated T cells.
SN of RPE eyecups from C57BL/6 mice were removed 24 hours after incubation with SFM and placed together with anti-CD3 antibody and T cells from either C57BL/6 or TGF-β RII DN mice for a further 24 hours. Positive control wells contained TGF-β RII DN T cells and anti-CD3 antibody and the negative control wells contained only TGF-β RII KO T cells.

A. T cell proliferation was measured at 48 hours.

B. The supernatants of culture wells were collected at 48 hours and used in IFN-γ ELISA. Bars represent the mean ± SEM of triplicate wells from a representative experiment.

* indicate mean values significantly greater than the positive control effect (p<0.05).
Figure 4.9

A

<table>
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</tr>
<tr>
<td>Negative Control</td>
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</tr>
<tr>
<td>C57Bl/6 T cells</td>
<td>6000</td>
</tr>
<tr>
<td>TGFβRII DN T cells</td>
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B

<table>
<thead>
<tr>
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<th>IFN-γ (ng/ml)</th>
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</thead>
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<tr>
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</tr>
<tr>
<td>Negative Control</td>
<td>0</td>
</tr>
<tr>
<td>C57Bl/6 T cells</td>
<td>*</td>
</tr>
<tr>
<td>TGFβRII DN T cells</td>
<td>*</td>
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</table>
4.6 Discussion

In these experiments, some of the molecular strategies that confer an immune privileged status on the subretinal space are identified. The emphasis was placed on the contribution of RPE to the immunosuppressive microenvironment of the subretinal space by studying the effect of soluble factors produced \textit{in vitro} by intact monolayer of RPE in eyecups on antigen and antibody activated T cells. Naïve T cells generally do not cross blood:retinal barriers, but activated T cells have been shown to readily cross the outer blood retinal barrier produced by tight junctions between RPE cells (Devine 1996). Having crossed the barrier, activated T cells can initiate inflammation and threaten the integrity of immune privilege. The results show that RPE inhibits T cell proliferation and prevents the activation of the pro-inflammatory Th1 subtype. Moreover the inhibitory effects of RPE on T cells are mediated by soluble factors since supernatant of the RPE eyecups was used in our assays. TGF-β, SOM and TSP are the major, if not the sole, soluble factors produced by RPE that are responsible for this effect.

Since RPE expresses mRNA for and secretes TGF-β and since active TGF-β₂ has also been found to be a potent inhibitor of T cell activation (Streilein 1999), it was logical to look for the presence of and investigate the role of TGF-β in T cell inhibition by RPE SN. Using a sensitive bioassay, it was demonstrated that latent TGF-β is produced constitutively by the monolayer of RPE and is present in the supernatant of cultured RPE eyecups. However latent TGF-β in RPE SN was unable to inhibit T cell proliferation; only upon activation did TGF-β mediate T cell inhibition. Additionally it was demonstrated that SN of RPE was unable to inhibit activation of purified T cells taken from TGF-β RII DN mice, illuminating the fundamental role that TGF-β plays in T cell inhibition. Other laboratories have shown previously that RPE is able to inhibit T cells. Liversidge \textit{et al.} have demonstrated that RPE cells use both soluble and membrane-bound mechanisms to suppress lymphocyte proliferation stimulated by antigen, mitogen, and IL-2 (Liversidge 1993). Since indomethacin restored the proliferative responses of lymphocytes to specific antigen and mitogens, these authors concluded that PGE₂ was a soluble constituent responsible in part for T cell suppression. Since the authors did not find TGF-β in the supernatant of co-cultured T cells and RPE by western blotting, they concluded that TGF-β did not play a role in T cell activation (Liversidge 1993). Rezai and colleagues subsequently corroborated the ability of
human foetal RPE (HFRPE) cells to suppress, via apoptosis, T cell activation across a transwell membrane. Their search for the factor secreted by HFRPE cells excluded TGF-β, TNF-α and IL-10 since these factors were not found by ELISA, and the addition of the factors to T cell cultures did not lead to T cell apoptosis (Rezai 1999). It is possible that in these studies, TGF-β was present but was latent or that an intact monolayer of RPE is essential for RPE production of appreciable levels of active TGF-β (Ishida 2003). It is noted that if the monolayer of RPE is disrupted by sodium iodate treatment, SN of RPE eyecup loses its inhibitory effects (unpublished data, manuscript in preparation).

In related experiments it was demonstrated that eyecups from mice with the thrombospondin 1 gene knocked out (TSP-1KO) were unable effectively to inhibit T cell activation. RPE SN from TSP-1KO animals contained comparable levels of latent TGF-β to that of wild type C57BL/6 eyecups but lacked active TGF-β. The mechanisms of TGF-β activation in the subretinal space are not fully understood. Activation of TGF-β in vitro is achieved by treating the latent TGF-β with extremes of heat and pH, whereas, in vivo the activation of TGF-β is enzymatic through proteolytic activities of plasmin, cathepsin and others (Lyons 1990). Thrombospondin binds the latent TGF-β complex and can activate it (Murphy-Ullrich 2000) and its effectiveness is usually enhanced in the presence of proteolytic enzymes. Although RPE produces plasminogen activators (Siren 1992) and displays receptors for urokinase (Elner 2002), there was no indication that RPE from TSP-1KO mice could mediate TGF-β activation through enzymatic mechanisms alone. Since the SN of eyecups from TSP-1KO mice were unable to inhibit T cell activation, a conclusion can be made that it is essential for TSP to be expressed by RPE to create and maintain the immunosuppressive microenvironment of the subretinal space, probably through its ability to activate latent TGF-β.

RPE was found to express mRNA for prosomatostatin and to secret somatostatin into RPE eyecup SN. RPE express receptors for many neuropeptides (Friedman 1991), and neuropeptides such as somatostatin, vasoactive intestinal peptide (VIP), substance P, β-endorphin, and calcitonin gene-related peptide (CGRP) have all been shown to play a part in proliferation and differentiation of cultured RPE (Kishi 1996). Some factors, such as insulin growth factors, function in an autocrine manner to stimulate proliferation, migration, and plasminogen activation by RPE (Grant 1990). Since SOM has been shown to be a component of AqH and to inhibit Th1 cell activation (Taylor 2003, Koh 2000), it was reasonable to...
examine RPE for SOM production. Since RPE possess receptors for SOM (Klisovic 2001), an autocrine effect is speculated for SOM. In these experiments, SOM was shown to inhibit Th1 activation, but to a lesser extent than that due to active TGF-β. Moreover TGF-β and SOM do not appear to act synergistically. Neuropeptides, such as SOM, can directly affect the cytokine secretion of T cells with or without coexistent antigen stimulation (Levite, 2000). However, these neuropeptides may activate the cells differently according to whether T cells are naïve or antigen activated. In the setting used in the above experiments, SOM inhibited Th1 cell activation. However, it is possible that SOM may behave differently under different physiological conditions.

The knowledge gained here demonstrates that privilege in the subretinal space of the normal eye is dependent, in part, on the expression of TSP and the production of immunosuppressive factors by RPE- TGF-β and SOM. Transplanted materials are, however, placed in diseased retinas and in the following chapter effects of RPE dysfunction on the ability of RPE to contribute to immune privilege status of the subretinal space is further studied.
Chapter 5 – Impact of RPE dysfunction on expressions of immune privilege in the subretinal space

5.1 Introduction

In the proceeding chapter, the role of retinal pigment epithelium (RPE) in down-regulating the adaptive immune response and the contribution that RPE monolayer makes to immune privilege of the subretinal space was discussed. RPE contributes to immune privilege of the subretinal space by: Preventing the onslaught of immune cells into the subretinal space by forming the outer blood retinal barrier; by expressing relatively low levels of the MHC molecules making it difficult for immune cells to recognise RPE and attack it; and by elaborating soluble factors such as TGF-β, somatostatin (SOM) and thrombospondin-1 (TSP-1), which were shown to inhibit T cell activation. Secretion of PGE2 and surface expression of CD95L by RPE have also been reported and both have been implicated in the ability of RPE to dampen the immune response (Liversidge 1993, Bellgrau 1995).

If the presence of immune privilege in the subretinal space is largely dependent upon the immunomodulatory and barrier properties of RPE, then one can hypothesise that RPE dysfunction may or should lead to loss of immune privilege. Immune privilege is believed to be an important physiological adaptation of the eye that prevents innate and adaptive immune responses from destroying precious ocular cells that are essential for vision and incapable of regeneration. Immune privilege also prevents immunologically induced inflammation from clouding the ocular media and distorting the visual axis, for when the ocular cells die and/or the visual axis is disrupted, loss of vision (blindness) is the inevitable consequence.

In light of evidence that immune privilege in the subretinal space is dictated in large measure by physical and functional properties of RPE, it is relevant to examine the relationship between functional integrity of intact monolayers of RPE cells and the existence of immune privilege in this space. To examine this issue, several genetically determined or experimentally created models in mice in which RPE is functionally abnormal or parameters of immune privilege in the subretinal space are lost, were chosen.
Sodium iodate treatment has been shown to selectively poison RPE cells and disrupt their tight junctions (Anstadt 1982). Moreover sodium iodate treated animals failed to support immune deviation in the subretinal space (Wenkel 1998). In the previous chapter it was demonstrated that thrombospondin-1 knockout (TSP-1KO) mice are unable to inhibit T cell activation through their inability to activate TGF-β. Here the presence of immune privilege in their subretinal space will be studied by investigating the ability of TSP-1KO mice to support immune deviation. Furthermore since RPE from TSP-1KO mice is unable to steer T helper cells away from Th1 phenotype, the role of TSP, and by extension active TGF-β, in autoantigen induced uveitis, a Th1-dependent inflammatory response is studied. Since the ultimate aim of studies here and elsewhere, is to facilitate retinal transplantation in the subretinal space of patients with retinal degenerations, the mouse model of retinal degeneration, the rd (C3H/HeN) mouse was chosen and the microenvironment of their subretinal space was further investigated.

5.2 Results

It was shown in the previous chapter supernatants from RPE eyecups of normal C57BL/6 mice suppressed profoundly OVA-stimulated DO11.10 T cell activation. It was also reported that SN obtained from RPE eyecups of TSP-1KO mice was far less efficient than the SN from normal C57BL/6 eyecups at inhibiting DO11.10 T activation. However, in the DO11.10 T cell activation assay, unfractionated lymph node cells were used as responders. Thus, both antigen presenting cells (APC) and T cells were present, either or both of which could be influenced by factors present in RPE eyecup SN. In order to determine the extent to which RPE-derived immunosuppressive factors act directly on T cells, we tested RPE SN on T cells activated in vitro by mitogenic anti-CD3 antibodies. In this assay, the responding T cells were purified from lymphoid cell suspensions, removing virtually all APC. Purified T cells prepared from spleens of normal C57BL/6 mice, were stimulated in vitro with anti-CD3 antibodies in presence of SN obtained from posterior eyecups prepared from normal C57BL/6, SI-pretreated C57BL/6, TSP-1KO and rd mice for 24 hours. 3H-thymidine was then added to the some wells and thymidine uptake was measured as a function of T cell proliferation. Some cultures were allowed to proceed for 48 hours and their supernatants were collected for IFN-γ measurement. Wells with T cells and anti-CD3 antibody and no SN...
served as positive controls and those containing only T cells in SFM served as negative controls.

Stimulated T cells mounted an appreciable proliferative response to anti-CD3 antibody (Figure 5.1). T cells stimulated by anti-CD3 in the presence of SN from normal C57BL/6 eyecup proliferated poorly. By contrast, eyecup SN from SI-pretreated and from TSP-1KO and rd mice were unable to suppress T cell proliferation (Figure 5.1). As demonstrated in Figure 5.2, stimulated T cells produced significant amounts of IFN-γ. By contrast, T cells stimulated in the presence of normal SN from C57BL/6 eyecups produced virtually no IFN-γ compared to SN from normal eyecups, the SN from eyecups with abnormal RPE was significantly weaker in suppressing IFN-γ production by stimulated T cells (Figure 5.2). These results reveal that factors produced by normal and abnormal RPE in eyecups directly suppress T cell activation, irrespective of their capacity to alter APC function. Moreover, abnormal RPE secretes factors that are more effective at inhibiting IFN-γ production than suppressing T cell proliferation.
Figure 5.1: Effects of SN of RPE eyecups from SI-pretreated, TSP-1KO, C3H/HeN and C57BL/6 mice on anti-CD3 stimulated T cell proliferation

24 hr SN of eyecups prepared from eyes of SI-pretreated, TSP-1KO, C3H/HeN (rd) and C57BL/6 mice were incubated with purified T cells and anti-CD3 antibody for 24 hours. \(^{3}\text{H}-\text{thymidine was added to the wells and T cell proliferation was measured a day later. Positive control represents wells containing T cells with anti-CD3 antibody and negative control denotes wells containing only T cells and SFM. \* indicates mean values significantly greater than C57BL/6 SN effect (p<0.05). Unlike the SN of eyecups from TSP-1KO, C3H/HeN and SI-pretreated mice, SN of RPE from C57BL/6 mice were able to significantly inhibit T cell proliferation.
Figure 5.1

- Positive Control
- Negative Control
- C57/BL/6 SN
- TSPKO SN
- C3H/HeN SN
- SI-pretreated SN
Figure 5.2: Effects of SN of RPE eyecups from SI-pretreated, TSP-1KO, C3H/HeN and C57BL/6 mice on IFN-γ production by anti-CD3 stimulated T cells

Purified T cells prepared from spleen of C57BL/6 were incubated with anti-CD3 antibody and 24h SN of RPE eyecups from eyes of SI-pretreated, TSP-1KO, C3H/HeN and C57BL/6 for 48 hours. The supernatant of the wells were then collected and used in IFN-γ ELISA. Positive control represents wells containing T cells with anti-CD3 antibody and negative control denotes wells containing only T cells and SFM. * Indicates mean values significantly greater than C57BL/6 SN effect (p<0.05).
Figure 5.2

- Positive Control
- Negative Control
- C57/BL/6 SN
- TSPKO SN
- C3H/HeN SN
- SI-pretreated SN
5.2.1 Ability of eyes with disturbed RPE to promote immune deviation

Since the SN of RPE eyecups produced from eyes of TSP-1KO, SI-pretreated, and rd mice were unable to inhibit T cell activation to the same degree as the eyecup of wild type C57BL/6 mice, it was of interest to see if the subretinal space of these mice is able to support the induction of immune deviation. SI-pretreated mice have already been shown to lack the ability to support ACAID in the subretinal space (Wenkel 1998). Ovalbumin was injected into the anterior chamber (AC) or the subretinal space of a group of TSP-1KO and a group of rd mice. A week later these mice and a group of naïve mice (positive control) from each strain received a subcutaneous injection of OVA and CFA. On day 14 all mice and a group of naïve mice from each strain, acting as negative control, were ear challenged with antigen and their ear thickness was measured by micrometer 24 hours later. Figure 5.3A demonstrates that TSP-1KO mice receiving a subcutaneous injection of OVA and CFA mounted a large delayed hypersensitivity (DH) response. In contrast to the wild type C57BL/6, TSP-1KO mice that were first received an inoculation of OVA in the subretinal space or the anterior chamber displayed intense DH responses. Delayed hypersensitivity was successfully induced in rd mice receiving a subcutaneous injection of OVA and CFA (positive control) (Figure 5.3B). C3H/HeN (rd) mice that had received intracameral injection of OVA in to the subretinal space prior to subcutaneous immunisation failed to demonstrate immune deviation (Figure 5.3B). Conversely, rd mice that had received OVA injection into the anterior chamber prior to receiving a subcutaneous immunisation with OVA and CFA, acquired anterior chamber associated immune deviation (ACAID) (Figure 5.3B).

The results demonstrate that lack of TSP production by RPE in TSP-1KO mice prevents their eyes from promoting immune deviation either via the anterior chamber or the subretinal space. Rd mice, with an abnormality primarily expressed in the posterior segment of the eye, failed to support immune deviation in the subretinal space although this property was retained in the anterior chamber.
Figure 5.3: Capacity of eyes of thrombospondin-1 knock out and C3H/HeN mice to support immune deviation to OVA antigen

A group of TSP-1KO mice (A) and a group of C3H/HeN mice (B) received injections of OVA into the anterior chamber or the subretinal space. Seven days later, the experimental animals, as well as naïve mice from each strain (positive control), received subcutaneous injections of OVA and CFA. On day 14 all mice as well as a group of naïve mice from each strain, acting as negative control, received ear challenge with OVA antigen. Their ear thickness was measured by micrometer 24 hours later. * indicates mean ear swelling responses significantly lower that positive control ($p<0.05$).
Figure 5.3

A

TSPKO

Ear Measurement (μm)

Positive Control

Negative Control

Anterior Chamber

Subretinal Space

B

C3H/HeN

Ear Measurement (μm)

Positive Control

Negative Control

Anterior Chamber

Subretinal Space

* Indicates significant difference.
5.2.2 Role of TGF-β in immune privilege of the subretinal space

It was shown in the previous chapter that the RPE from TSP-1KO mice was unable to activate TGF-β and that this inability correlated with the loss of RPE-mediated inhibition of T cell activation. It was of interest to see if such a correlation also exists for SI-pretreated and rd mice. RPE eyecups were prepared from eyes of C57BL/6 mice that received an i.v injection of sodium iodate 48 hours previously, and from rd mice. SN harvested from these eyecups after 24 hr incubation were diluted 1:10, and divided into two portions. One portion was assayed for presence of active TGF-β and the other portion was transiently acidified and assayed for total TGF-β using the mink lung cells bioassay. The results presented in Figure 5.4 indicate that SN of RPE eyecups of both rd and SI-pretreated mice contain appreciable amount of latent TGF-β. SN of RPE eyecups from rd mice contained comparable levels of active TGF-β to that of eyecups from normal C57BL/6 mice. By contrast, SN of eyecups from SI-pretreated animals contained significantly lower amounts of active TGF-β (Figure 5.4), than eyecups from untreated controls. Thus it appears that SI-treated and TSP-1KO mice in which the dysfunction of RPE is the primary abnormality, lose the ability to successfully activate TGF-β. Even though rd mice have retinal abnormalities, their RPE is sufficiently functional to allow activation of TGF-β.
Figure 5.4: Total and active TGF-β content of SN of RPE eyecups prepared from C57BL/6, sodium iodate-pretreated and C3H/HeN mice

RPE eyecups were prepared from C57BL/6, SI-pretreated and C3H/HeN mice, and SN were collected after 24 hours of incubation in SFM. Mv1Lu cells from sub-confluent cultures were incubated with unmodified or acid treated SN. \(^3\)H-thymidine was added to each well and four hours later the amount of incorporated label was measured.

A = Active TGF-β, T= Total TGF-β. * indicates mean values significantly greater than C57BL/6 SN effect (\(p<0.05\)).
Figure 5.4

- C57BL/6
- Sl-pretreated SN
- C3H/HeN mice
5.2.3 Development of experimental autoimmune uveoretinitis in C57BL/6 and Thrombospondin-1 knock out mice

The role of TSP and active TGF-β in the ability of C57BL/6 mice to withstand autoantigen-induced inflammation was explored next. Since experimental autoimmune uveitis (EAU) is mediated by Th1 cells, and since we found the RPE from TSP-1KO mice did not inhibit IFN-γ production by activated Th1 cells, a hypothesis was made that TSP-1KO mice might experience exaggerated EAU compared to controls. Both C57BL/6 and TSP-1KO mice were immunised subcutaneously with IRBP peptide and CFA. The mice were also given pertussis toxin (PTX) intraperitoneally as an additional adjuvant. Their retinas were examined clinically at regular intervals and the degree of uveitis was measured and scored (Figure 5.5). Wild type C57BL/6 mice displayed significant retinal inflammation with the average maximal score reaching 2.5 ± 0.6 on day 18 after immunisation (Figure 5.6, circles). 120 days after immunisation, the disease abated and eyes reached the score of zero. TSP-1KO mice, on the other hand, had a significantly higher average score of 3.5 ± 0.5 at the peak of their EAU and these mice continued to display severe inflammation throughout the examination period (120 days) (Figure 5.6, squares). Our results indicate that TSP is essential in diminishing the severity and duration of uveitis in EAU. At day 140 after immunisation, all animals were sacrificed and their eyes were processed for histology.
Figure 5.5: Clinical scoring system for the degree of uveitis

The degree of uveitis was scored based on vessel dilatation; number of white, focal, perivascular lesions; and the extent of retinal vessel exudates, haemorrhage, and detachment. Welling responses significantly lower than positive control \( (p<0.05) \).
Figure 5.5

Score 0
No evidence of

Score 1
Focal vasculitis <5
Soft exudate spotted <5

Score 2
Linear vasculitis of within half of retina
Soft exudate spotted in half of retina

Score 3
Linear vasculitis over half of retina
Soft exudate spotted over half of retina

Score 4
Retinal haemorrhag in addition to vasculitis

Score 5
Exudative retinal detachment or subretinal haemorrhag
Severe exudate in addition to vasculitis
Figure 5.6: Induction of experimental autoimmune uveoretinitis (EAU) in C57BL/6 and TSP-1KO mice

Groups of C57BL/6 and TSP-1KO mice were immunised subcutaneously with IRBP peptide and CFA and received simultaneously intraperitoneal PTX. Their retinas were examined through dilated pupils at regular intervals. The extent of uveitis is represented as the mean EAU score for both eyes on the day of examination.
Figure 5.6

- TSP KO
- C57BL/6

Mean EAU score vs. Days after immunisation
5.2.4 Retinal recovery of C57BL/6 and thrombospondin-1 knockout mice from experimental autoimmune uveoretinitis

To examine the state of the retina in recovered C57BL/6 mice and contrast it with that of TSP-1KO mice, immunised mice from both groups were sacrificed at 140 days after initial immunisation, their eyes were enucleated, fixed in 10% formalin, embedded in methacrylate, sectioned and stained with H&E. Typical example of the results are depicted in Figure 5.7. Figure 5.7A demonstrates a retina taken from a naïve TSP-1KO mouse. All retinal layers are present in normal thickness and configurations with an intact RPE layer. The retinas of C57BL/6 mice that recovered from EAU, retained normal retinal architecture, although minor irregularities were detected over a small section of the RPE and slight thinning of the outer and inner nuclear layers (Figure 5.7B). By contrast, the retina of immunised TSP-1KO mice displayed severe abnormalities (Figure 5.7C): the RPE layer was discontinuous and in fact totally destroyed in parts. There were minimal photoreceptor outer segments remaining and the inner nuclear layer was reduced to half of its original thickness. There was a complete loss of the outer plexiform layer with disorganisation of the inner plexiform and the ganglion cell layers. There were areas of haemorrhage within the outer retina, and new vessels were seen to stretch between the ganglion cell layer and the inner nuclear layer and between the choroid and the photoreceptor layer (Figure 5.7 d and e).

The results reveal that remarkable recovery from inflammation occurs in the retinas of C57BL/6 mice that recover from EAU. Alternatively mice deficient in TSP displayed a devastating and prolonged inflammatory response that destroyed the organisation of the retina and failed to resolve.
Figure 5.7: Histology of eyes from C57BL/6 and TSP-1KO mice 140 days after induction of EAU

A. Retina of a naïve TSP-1KO mouse (X20).

B. Retina of C57BL/6 mouse at day 140 after induction of EAU at which time the clinical uveitis score was 0 (X20). Punctate neuron loss is noted in both inner nuclear and photoreceptor cell layers. The normal architecture of the retina is preserved.

C. Retina from TSP-1KO mouse 140 days after IRBP immunisation at which time the average clinical uveitis score was 3 (X20). The RPE layer appears discontinuous and is totally destroyed in parts (arrows). Photoreceptor outer segments (OS) are barely visible; there is complete loss of the outer plexiform layer, and profound disorganisation of the inner plexiform layer (IPL) and ganglion cell layer (GCL). An area of haemorrhage (short arrow) and a new vessel (arrow head) are seen to stretch between the ganglion cell layer and the inner nuclear layer.

D. and E. A higher magnification of TSP-1KO retina 140 days after immunisation. Two areas of new vessel formation are evident: one from the choroidal circulation incorporating the RPE (D), and the other from the retinal circulation (E).
Figure 5.7 A and B
Figure 5.7 D and E
5.3 Discussion

The existence of immune privilege in the subretinal space offers hope for the eventual success of transplants of allogeneic retinal tissues in order to restore vision. To what extent this immune privilege is maintained in the presence of retinal diseases, for which transplantation is indicated, is important to understand. To approach this issue we have selected to study three pathological conditions in which the RPE alone or in combination with the neural retina is abnormal. Our findings demonstrate that soluble factors produced by ex-vivo cultures of RPE monolayer from eyes of TSP-1KO, sodium iodate treated, and rd mice are less able to inhibit T cell activation in vitro as compared to cultures of C57BL/6 wild type control RPE. The subretinal space of C3H/HeN mice with retinal degeneration, of TSP-1KO mice, and of mice pretreated with sodium iodated failed to support induction of OVA-specific immune deviation. Moreover, TSP-1KO mice that received an uveitogenic regimen of IRBP experienced significantly enhanced uveitis that failed to resolve.

Experiments performed on RPE eyecups from SI-pretreated animals testify to the potential of RPE to produce an immunosuppressive microenvironment in the subretinal space. SI-pretreatment leads to loss of tight junctions between RPE cells and loss of the outer BRB (Anstadt 1982). Our laboratory has shown previously that eyes of SI-pretreated mice fail to support immune deviation via the subretinal space (Wenkel 1998). In a related study, SI treatment was shown to promote the induction and development of uveitis in Brown Norway rats, a strain that is usually resistant to development of EAU (Tanaka 1991). It is important to know whether it is the disruption of the outer BRB caused by SI-pretreatment that leads to the loss of immune privilege in the subretinal space, or the specific SI- associated toxicity of the RPE that is responsible for loss of immune deviation in the subretinal space of SI-pretreated animals.

Partial to complete breakdown of the outer BRB occurs in many retinal diseases for which transplantation may someday be a therapy. The surgical procedure by which the transplanted material is placed into the subretinal space is itself able to cause breakdown of the inner and/or outer BRBs. Beyond inducing trauma, surgical intervention is associated with release of pro-inflammatory mediators and the expression of inflammation. To this end, it has been shown that intravitreal injection of IFN-γ in rats and rabbits induces leukocytic influx into the retina (Lee 1990, Hjelmeland 1992) through a breach in the BRB (Martiney 1992). Therefore, it is logical to suspect that breakdown of the BRB after SI treatment causes
the local loss of immune privilege. Streilein and co-workers, however, have recently demonstrated that the presence of an intact blood:ocular barrier is not essential for persistence of immune privilege in the anterior chamber (Streilein 2003). In our experiments, we have demonstrated that the SN of RPE eyecups from SI-pretreated animals was significantly less able to inhibit T cell activation. Moreover, the SN from these eyecups contained far less active TGF-β than did eyecup SN from untreated animals. Thus, we consider it possible that the inability of the subretinal space of SI-pretreated animals to support induction of immune deviation may have more to do with the ability of their RPE to produce an immunosuppressive microenvironment than to their retention of an intact BRB.

Retinal degeneration (rd) mice were chosen so that the presence of immune privilege could be evaluated in eyes with spontaneous disease. As these mice age, they display a partial loss of RPE leading to loss of the outer BRB (Neuhardt 1999). Here we have shown that the explanted RPE of rd mice were unable to inhibit T cell proliferation in vitro and were much less able to suppress IFN-γ production. Moreover, young rd mice proved incapable of promoting immune deviation in the subretinal space even though they retained this ability in the anterior chamber. This differential loss of ACAID in the subretinal space and not in the anterior chamber of SI-pretreated animals has been shown previously by Wenkel et al. (Wenkel 1998). It is of interest that Wenkel and his co-workers also showed that if immune privilege in the anterior chamber was abrogated by application of light cauteryisation to the central corneal surface, immune deviation could not be evoked by antigen placed in the subretinal space. This finding implies that a functional link connects these two intraocular compartments, and that the anterior chamber plays a more dominant role in dictating the immune privileged outcome. Other studies have reported that the anterior chamber of aged rd mice no longer supports ACAID induction (Welge-Lüssen 1999). Therefore, as the retinal degeneration proceeds in aging rd mice, the failure of their RPE to generate pertinent immunosuppressive factors robs first the posterior, and then the anterior compartments of ocular immune privilege.

It can be concluded that under normal circumstances RPE secretes immunosuppressive factors that modulate both induction and expression of adaptive immunity to antigens derived from the subretinal space, and that this raises the possibility that RPE could modulate induction and expression of immunopathogenic autoimmunity to retinal and uveal tract autoantigens.
TGF-β is an important immunosuppressive molecule in the eye (Streilein 1999), and this cytokine is produced by RPE (Tanihara 1993). We have shown in the previous chapter that the conversion of latent TGF-β to its active form in the subretinal space is reliant almost exclusively upon the presence of TSP, a molecule also produced by RPE. Our findings in TSP-1KO mice that were immunised with IRBP reveal that the absence of TSP, and therefore failed activation of TGF-β, resulted in potentially destructive and sustained autoimmune inflammation of the uveal tract and retina.

Hara et al have shown previously that pre-emptive induction of IRBP-specific ACAID can prevent the onset of EAU, and that induction of ACAID once EAU is established abruptly terminates EAU (Hara 1992). Others have shown that regulatory mechanisms come into play as EAU wanes following its initial clinical expression (Taylor 2002). Since we also demonstrate that the subretinal space of TSP-1KO mice does not support immune deviation, we infer that induction of immune deviation is responsible, at least in part, for the suppression of autoimmunity that normal mice use to limit both the extent and the destruction of EAU. Unlike the retina of normal C57BL/6 mice, the retina of TSP-1KO mice subjected to EAU revealed new vessel formation from both the choroidal and retinal circulations. TSP-1 and TSP-2 have been shown to inhibit angiogenesis in vivo and in vitro assays (Tolsma 1993). Moreover, over-expression of TSP-1 or -2 in tumour cells leads to inhibition of angiogenesis and tumour growth (Bleuel 1999, Streit 1999). Peptides derived from TSP-1 were also shown to inhibit endothelial cell growth in a retinal explant assay (Shafiee 2000). The antiangiogenic activity of TSP has been attributed by various investigators to its ability to activate latent TGF-β (Schultz-Cherry 1995), to bind to CD36 (Dawson 1997), or to interact with heparan sulfate proteoglycans (Guo 1992). The presence of new vessels in the retina of TSP-1KO mice that had developed uveitis points to an important role that TSP plays, probably through activation of TGF-β, as an anti-angiogenic factor in the retina.

From the results of the above experiments it can be concluded that the capacity of the subretinal space to support induction of immune deviation is dependent on the integrity of the outer BRB, and on the ability of the RPE to produce active TGF-β. If either of these features is deficient, the immune deviation component of immune privilege in the subretinal space is degraded. This may be potentially worrisome if one aims to place grafted materials into the subretinal space of diseased eyes. However, even though immune deviation was lost in the subretinal space of rd mice, they were still able to inhibit T cell activation and deviate the
immune system away from the pro-inflammatory course. So in the younger rd mice at least, this ability may explain the reason why transplanted materials survive for an extended time in the subretinal space of rd mice and why they may eventually be rejected. In general, presence of immune privilege in the subretinal space is dependent in part by the integrity and functionality of an RPE monolayer.
Chapter 6 – Retinal pigment epithelium modulates function of innate immune cells

6.1 Introduction

The efferent phase of the cellular immune system involves reactivation of T cells that have entered the site of injury to produce pro-inflammatory cytokines. These cytokines, in turn, recruit innate immune cells and set up an inflammatory response. The innate immune cells, neutrophils, macrophages and NK cells identify pathogens through surface recognition and become activated. NK cells lead to direct lysis of pathogens, and neutrophils and macrophages secret toxic O₂ and nitrogen compounds leading to intense inflammation. In addition, innate immune cells produce inflammatory cytokines and chemokines, leading to recruitment of additional leucocytes and further exacerbation of inflammation. During inflammation in the subretinal space either due to a surgical procedure that transplants grafts at this site, or during uveitis, the blood: retinal barrier that usually protects the retina from entry of innate immune cells, is disrupted. Inflammation associated with the injury to the subretinal space also releases IFN-α, β and IL-1β, which can induce RPE to recruit innate immune cells by producing monocyte chemotactic protein (MCP) (Elner 1991), neutrophil chemotactic factor (IL-8) (Elner 1990).

Before discussing interactions between the RPE and innate immune system, some of the members of the innate immune system are introduced further.

6.1.1 Neutrophils: A brief overview of the cells, their locations, and their functions

Neutrophils are the most common type of leucocytes in the human blood (3000-6000 per μl of blood; 40-75% of leucocytes). They are characterised by 3 distinct features as seen by electron microscopy: Multi lobed nucleus; numerous membrane bound granules in the cytoplasm with a large round primary granule, and specific granules which are smaller, denser and more numerous. All other cytoplasmic organelles are scarce. Neutrophils are the principal cells involved in the acute inflammatory response. They are highly mobile and migrate from small blood vessels to sites of tissue damage where they engulf and destroy cell debris and microorganisms by phagocytosis. Through release of granule products and
cytokines, they act as important effector cells. Neutrophils can be recruited by both the immune mechanisms (via Fc receptors, complement and anaphylatoxin receptors) and by non-immune mechanisms such as formyl peptides through poorly identified receptors (Cousins 1996). Since they possess very few organelles, neutrophils are incapable of continuous function and degenerate through apoptosis after a single burst of activity.

Neutrophils are the main infiltrating cells in bacterial infection of conjunctiva, cornea, sclera and vitreous as well as in herpes simplex keratitis and retinitis as well as being the principal cell type in LPS-induced and cytokine-induced inflammation (Green 1986, Cousins 1989, Niemialtowski 1992, de Kozak 1981). They have also been described in ocular inflammatory conditions such as phacoanaphylactic endophthalmitis (Marak 1976), which may occur following cataract surgery. During the acute phase of T-cell mediated delayed hypersensitivity reactions, neutrophils predominate. Neutrophils are prominent in allergic uveoretinitis induced by S antigen and in animal models of immune complex activated keratitis and uveitis (de Kozak 1981). Thus neutrophils dominate ocular inflammation regardless of the cause.

Neutrophils are released into the circulation in inactive form but become activated by chemo-attractants, leading to various cell surface changes that increase the adherence of neutrophil. These activated neutrophils adhere to and migrate through the endothelium and enter the tissue. Once at the site of inflammation, granular products of neutrophils such as protease, elastase and collagenase, are released contributing and amplifying inflammation. Neutrophils also release chemoattractants and activators of complement recruiting other innate immune cells, and with the help of their granular content of lysosomal enzymes and oxygen products, phagocytose antigenic materials.

6.1.2 Monocytes: A brief overview of the cells, their locations, and their functions

Monocytes are the largest of the white blood cells and constitute 2-10% of leucocytes in the human blood (100-700/μl). Monocytes possess a large indented nucleus and an extensive cytoplasm filed with small lysosomes, a well-developed Golgi apparatus and numerous mitochondria. The accumulation of blood monocytes at a site of inflammation is a common feature in many inflammatory conditions, including rheumatoid arthritis. Monocytes differentiate into macrophages in the tissues and are believed to contribute to the inflammatory reaction. Macrophages have three primary functions: they act as an
inflammatory effector cells by phagocytosing the offending pathogens and dead cells; they are one of the main antigen presenting cells for T lymphocytes; and regulate other processes such as fibrosis.

They are activated by bacterial toxins, by exposure to inflammatory mediators such as IFN-γ and IL-1, and by phagocytosis of complement or antibody-coated pathogens. Once fully activated, macrophages kill and degrade phagocytosed pathogens as well as produce a variety of pro-inflammatory cytokines such as IL-1, IL-6, TNF-α and chemokines, thus recruiting more leucocytes and further increasing the inflammation. Macrophages are found in both acute and chronic inflammations in the ocular environment and at some sites can undergo morphologic changes to become epitheloid cells or multi-nucleated giant cells (Sheffield 1990). Recently the lab of Hendricks identified numerous CD45⁺ cells with dendriform morphology and CD11b co expression that within the pericentral and central region of the corneal stroma. 30% of these cells also co-expressed MHC class II (Brissette-Storkus 2001).

Since both neutrophils and macrophages use phagocytosis as a method for elimination of pathogens, the process of phagocytosis is briefly described.

### 6.1.3 Phagocytosis: the bare essentials

Killing of pathogens, removal of debris and clearance of antigenic materials are most efficiently carried out by phagocytosis, which is specific and receptor mediated. Antigens coated with Igs or activated components of complement bind to the Fc and complement receptors, amongst others, on the surface of phagocytes and are ingested. The process of ingestion is a multi step process beginning with membrane and lysosomal enzyme synthesis, formation of reactive O₂ and nitrogen intermediates and migration of granules to phagosomes. The process of phagocytosis takes place through: the presence of antimicrobial polypeptides present in cytoplasmic granules such as defencins and cathepsin G in the primary granules of neutrophils, and lysozyme and lactoferrin in the secondary granules of neutrophils; generation of reactive oxygen radicals produced during respiratory burst; and production of reactive nitrogen radicals. Oxygen free radicals are thought to mediate retinal injury during EAU (Rao 1990) and have a role in lens-associated uveitis (Marak 1992). IFN-γ and TNF-α in combination with LPS promote and TGF-β inhibits NO production. Although these products are designed primarily to kill the invading pathogens, they can also
indiscriminately damage bystander cells and contribute to tissue damage. In addition leukocytes can release or secret their contents such as perforin to cause further inflammation, cell lysis and tissue damage (Podack 1992).

6.1.4 A brief description of dendritic cells, macrophages and microglia in the retina and uvea

There is an extensive network of MHC class II positive dendritic cells and MHC class II negative ED2+/ED1+ macrophages in the iris stroma, ciliary body and choroid of rats and mice. McMenamin quantified dendritic cells and macrophages in the mouse uveal tract and found 80% of F4/80+ cells to be of macrophage lineage and 2/3 of class II positive cells of DC lineage. He also found the uveal tract to be weakly stained with B7.1, B7.2 and β2 integrins (McMenamin 1999). Forrester et al. have localised and characterised class II positive cells in the posterior segment of the eye. They have divided these cells into three groups: classical dendritic cells with high class II MHC expression; resident macrophages negative for class II MHC but with dendriform appearance; and blood borne ED1+ macrophages arriving into the posterior segment from the vasculature (Forrester1994).

Microglia in the retina of adult humans is a heterogeneous cell population, some with more similarity to macrophages and others to dendritic cells. They seem to enter the retina prior to vascularisation of the retina in the embryonic period. Provis et al. argue that microglia, which are positive for macrophage antigen, become established in the perivascular areas of the retina and those that are macrophage-Ag negative but MHC class II positive, representing DC, become the ramified microglia in adult retina (Provis 1996).

Ma and Streilein studied the contribution of resident retinal Microglia to the outcome of intraocular neural retinal grafts in the mouse model. They discovered that microglia are present at the time of grafting of the tissue and that in both syngeneic and allogeneic transplantation their number increased 12 days after grafting mainly within the centre of rosettes. In the allografts, they became activated and had high expression of class II MHC (Ma 1998). Ng and Streilein later found that the subretinal space of albino BALB/c mice contained a large number of resident inactive (5D4+, SG-) microglia whereas in pigmented mice, the cells were confined to the inner retinal layers. BALB/c mice raised in the dark had a relatively few 5D4+cells in the subretinal space but light exposure leads to a rapid
accumulation of 5D4+ cells at this site. The authors further showed that microglia in the subretinal space augment the function of RPE in phagocytosing ROS (Ng 2001).

### 6.1.5 NK cells

Natural killer (NK) cells, first recognised in mice due to their ability to lyse tumour cell targets (Kiessling 1975), are large lymphoid cells containing prominent intracellular granules making up 5-15% of the peripheral blood lymphoid cells and have similar frequencies in the spleen. They have also been found in the lungs (Puccetti 1980), liver (Wisse 1997), GI tract (Shanahan 1987) and uterine deciduas particularly in pregnancy. NK cells lack the surface markers for B or T cells and are present in mice genetically deficient in T and B lymphocytes (Kiessling 1975, Dorshkind 1985, Mombaerts 1992). They also lack CD4, the marker for T helper cells and only half of the NK cells in human and rats express the α/α form of CD8, the marker for cytotoxic T cells (Seaman 2000). The CD8 marker is lacking on mouse NK cells. By using CD16, a receptor for IgG, NK cells recognise and kill antibody-coated cells, a process called Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC).

NK cells produce a variety of cytokines including IFN-γ, TNF-α, TGF-β, and GM-CSF (Handa 1983, Cuturi 1989, Degliantoni 1985, Gray 1994). Their functions include protection against malignancy (Seaman 1987, Biron 1999) and viral infection, and regulation of hematopoiesis. NK cells do not possess any antigen-specific receptors but are able to recognise and kill a limited number of abnormal cells. Their anti infective function is mediated by their early arrival in viral infection and their ability to lyse the viral infected cells directly. Depending on the infective agent, NK cells can promote a Th1 response through release of cytokines such as IFN-γ, (Perrit 1998) but they can also be stimulated to secrete IL-5 leading to a Th2 response (Walker 1998).

NK cells may play an important role in autoimmunity by virtue of cytokine release and by circumstantial evidence cited in the literature: NK cells inhibit autoimmune response of CD4+ cells in the mouse model of colitis (Fort 1998); C57BL/6 mice depleted of NK cells are more susceptible to the induction of experimental autoimmune encephalitis (Zhang 1997); and patients with autoimmune diseases have been shown to have either an increased or a decreased level of NK cells in their peripheral blood (Sibbitt 1985, Negishi 1986, Garcia-Suarez 1993).
NK cells induce cytotoxicity by: Secreting perforin that produces holes in the target cells and allows entry of granzymes A and B; by inducing apoptosis through expression of TNF-related apoptosis-inducing signal (TRAIL); and by secretion of FasL when activated. NK cells are prevented from lysing self since the MHC class I antigens, present on almost all nucleated cells, bind receptors on NK cells and deliver inhibitory signals that block NK cell activation (Seaman 2000).

RPE holds a unique position, both anatomically and functionally, to modulate the presence and function of innate immune cells. RPE, on one hand, is known to employ strategies to prevent or contain inflammatory responses. Retinal pigment epithelium inhibits T cell activation and steers pro-inflammatory Th1 cells away from production of pro-inflammatory cytokines, which may prevent the recruitment of innate immune effectors. In the previous chapter, it was demonstrated that C57BL/6 retina that undergoes EAU in response to IRBP recovers and at least morphologically resembles normal retina. Since there is a massive influx of neutrophils and macrophages into the uvea and retina during the course of EAU, one would have expected a more severe and permanent destruction of retina. This lack of substantial retinal damage begs the question of whether there are immunomodulatory mechanisms in place to prevent the innate immune cells from producing an escalating destruction.

On the other hand, RPE not only is potentially capable of recruiting further innate immune cells to the site of inflammation but may also trigger their activation. RPE in human was found to constitutively express mRNA and secret protein for CD14, the receptor for LPS-binding protein (Elner 2003). This property of RPE can increase the inflammatory ability of LPS. RPE also constitutively expresses CD95L, which although capable of inhibiting Fas+ T cells, can trigger neutrophil and macrophage activation leading to additional recruitment of these cells.

There is evidence that innate immune privilege exists in the anterior segment of the eye. Apte and Niederkorn demonstrated that even though corneal endothelial cells are vulnerable to lysis by NK cells, aqueous humour inhibited NK cell-mediated cytotoxicity of both corneal endothelial cells and YAC-1 target cells preventing spontaneous lysis of corneal endothelial cells (Apte 1996). Chen et al. (Chen 1998) transfected CT26 cells (a colon carcinoma cell line) with CD95L and demonstrated that presence of Fas L on these cells led to their destruction by neutrophils once they were injected into mice. They also showed that if
the transfected tumour cells were incubated with bovine aqueous humour (AqH), there was significant inhibition of killing, demonstrating that components of aqueous humour strongly inhibited CD95L dependent activation of neutrophils partially through TGF-β. Both TGF-β₂ and α-MSH constituents of aqueous humour were shown to inhibit neutrophil-mediated killing of corneal endothelial cells (Streilein 2000).

Whether innate immune privilege exists in the subretinal space and if so, knowing the strategies employed by the eye to achieve this aim is essential to determine. In the following experiments the presence of innate immune privilege in the subretinal space is investigated by studying the effect of SN of RPE eyecups on the activation level of neutrophils and macrophages as well as on NK cell cytotoxicity. The results strongly point to the presence of innate immune privilege in the subretinal space.

6.2 Results

6.2.1 Effect of SN of RPE eyecups on neutrophil activation and role of TGF-β and SOM

Neutrophils can gain entry into the subretinal space during inflammatory processes involving the retina. Once activated, neutrophils perform two major functions: 1) phagocytosis of debris and release of toxic products such as reactive oxygen intermediates and 2) production of pro-inflammatory cytokines and chemokines to recruit additional inflammatory cells including more neutrophils. Since AqH has been shown to inhibit IL-1β production by neutrophils (Gregory 2002), we were interested to see if the SN of RPE eyecup can similarly inhibit IL-1β production. Neutrophils were obtained from C57BL/6 mice by casein stimulation. Casein-elicited neutrophils were incubated in vitro, for 18 hours, with 1:10 diluted SN of RPE eyecup produced from eyes of C57BL/6 mice. Casein-activated neutrophils incubated with SFM alone served as a positive control.

As depicted in Figure 6.1, casein-activated neutrophils produced appreciable amounts of IL-1β. Addition of SN of RPE eyecups significantly inhibited IL-1β production. Since TGF-β was found to be the component of the AqH responsible for inhibition of neutrophil activation (Chen 1998), in some wells, anti-TGF-β-antibody was added to ascertain the role of TGF-β in RPE inhibition of IL-1β production. SOM was shown to be partially responsible for inhibition of T cell activation in both AqH and SN of RPE eyecup (chapter 4).
for SOM was, therefore, added to some wells to determine the role it might play in neutrophil inhibition. Figure 6.1 demonstrates that blockage of both TGF-β and SOM by their respective antibodies led to the reversal of SN induced inhibition of IL-1β production by neutrophils. The results indicate that RPE by producing soluble factors significantly inhibits neutrophil activation. This inhibition is mediated through a TGF-β and SOM mechanism.

6.2.2 Effect of SN of RPE eyecups on IL-1β production by LPS-activated macrophages

Macrophages are found in all inflammatory responses and serve dual roles by both increasing inflammation- by producing toxic intermediates, by recruiting further inflammatory cells, and by presenting antigens to T cells- and helping the resolution of inflammatory responses by phagocytosing damaged cells and debris. In order to ascertain macrophage activation and its modulation by RPE, 5 x 10^5 RAW macrophage cells were incubated with diluted SN of RPE eyecup and LPS. As a positive control, macrophages were incubated with LPS alone. In some experiments, anti-TGF-β or anti-SOM antibodies were added to the cultures to assess the role of these soluble factors in activation of macrophages. Macrophages treated with LPS produced appreciable amounts of IL-1β as compared to untreated macrophages (Figure 6.2). Addition of SN of RPE eyecup significantly inhibited IL-1β production by LPS-activated RAW macrophage cell line. Addition of either anti-TGF-β or anti-SOM antibodies to the co-culture of RPE SN and RAW cells, failed to reverse SN induced inhibition of IL-1β production (Figure 6.2). The results indicate that soluble factors produced by RPE are able to inhibit macrophage activation and that neither TGF-β nor SOM contribute to this inhibition.
Figure 6.1: Effect of SN from C57BL/6 RPE eyecups on IL-1β production by neutrophils and role of TGF-β and SOM.

24 hours after incubation of RPE eyecups with SFM, SN was collected and diluted 1 in 10.

A. Casein activated neutrophils were incubated either alone (positive control) or with diluted SN of RPE. Other wells included LPS in addition neutrophils or LPS + neutrophils and SN of RPE eyecup for 18 hours at 37°C.

B. Casein-activated neutrophils incubated with SFM constituted the positive control. Experimental well included wells containing neutrophils and SN of RPE eyecups. To some of the experimental wells, anti-TGF-β or anti-SOM antibodies, alone or in combination, were also added. The wells were incubated in serum free media for 18 hours at 37°C.

Supernatants of the wells were removed and used in measurement of IL-1β production by ELISA. Bars represent the mean ± SEM of triplicate wells from a representative experiment. * Indicate mean values significantly less than the positive
Figure 6.1

A

IL-1β (pg/ml)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-TGF-β Ab</td>
<td>100</td>
</tr>
<tr>
<td>Anti-SOM Ab</td>
<td>150</td>
</tr>
<tr>
<td>anti-TGF-β Ab + anti-SOM Ab</td>
<td>200</td>
</tr>
</tbody>
</table>

B

IL-1β (pg/ml)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-TGF-β Ab</td>
<td>150</td>
</tr>
<tr>
<td>Anti-SOM Ab</td>
<td>200</td>
</tr>
<tr>
<td>anti-TGF-β Ab + anti-SOM Ab</td>
<td>250</td>
</tr>
</tbody>
</table>

Legend:
- Casein-activated neutrophils (CAN) in SFM
- SN of RPE eyecup + CAN
- LPS + CAN
- LPS + SN of RPE eyecup + CAN
Figure 6.2: Effect of SN from C57BL/6 RPE eyecups on IL-1β production by LPS-activated RAW cell macrophages and role of TGF-β and SOM.

24 hours after incubation of RPE eyecups with SFM, SN was collected and diluted 1 in 10.

Experimental wells contained diluted SN of RPE eyecup together with LPS and RAW cell macrophages. To some experimental wells anti-TGF-β or anti-SOM antibodies were also added to assess the role of TGF-β and SOM in IL-1β by macrophages. Wells containing RAW cells and LPS represented the positive control and those containing only RAW cells represented the negative control. The cultures were incubated in SFM for 24 hours at 37°C.

Supernatant of the wells were removed and used in measurement of IL-1β production by ELISA. Bars represent the mean ± SEM of triplicate wells from a representative experiment. ** indicate mean values significantly less than the positive control effect (p<0.01).
Figure 6.2

![Graph showing IL1-β (pg/ml) levels for Positive Control, Negative Control, and SN of RPE eyecup with anti-TGF-β Ab and anti-SOM-Ab.](image)

- Positive Control
- Negative Control
- SN of RPE eyecup
6.2.3 Effect of SN of RPE eyecup on nitric oxide production by activated macrophages

NO is a gas with various biological functions such as cytotoxicity, vascular relaxation and signal transduction. Different isoforms of nitric oxide synthetase (NOS) exist. cNOS is the constitutively produced isoform and participates in neurotransmission and vascular relaxation. The inducible form (iNOS) is produced by macrophages and neutrophils after LPS or cytokine stimulation and mediates the cytotoxic effect of macrophages. Taylor et al. have demonstrated that aqueous humour inhibited production of nitric oxide (NO) by IFN-γ and LPS-activated RAW cells through its content of CGRP (Taylor 1998). RPE cells express iNOS and produce NO after treatment with TNF-α or IFN-γ and LPS. Since RPE cells are capable of NO production, it was important to ascertain if soluble factors produced by RPE can also inhibit NO production. RAW cell macrophages were co-cultured with LPS and diluted 24-hour SN of RPE eyecup in serum free and phenol free medium for 24 hours at 37°C. Anti-TGF-β or anti-SOM antibodies were added to some wells to assess the role of these factors in NO production by LPS-activated macrophages. Positive control wells contained RAW cells and LPS and negative control wells contained only RAW cells. As demonstrated in Figure 6.3, SN of RPE contained only a very small amount of NO. RAW cells treated with LPS produced an appreciable quantity of NO. Addition of SN of RPE eyecup significantly potentiated this effect. Blocking TGF-β by using anti-TGF-β antibody significantly reversed the potentiating effect of the RPE SN, but did not inhibit NO production by LPS-activated RAW cells. Addition of anti-SOM antibody severely inhibited NO production by LPS-activated macrophages. To see if SOM alone can induce NO production, recombinant SOM at a concentration of 2μg/ml was added to the culture of macrophages. As seen in Figure 6.3, addition of SOM alone did not induce NO production. However, in the presence of LPS, NO production by RAW cells reached the positive control levels. The results imply that SN of RPE eyecups are unable to inhibit NO production by LPS-activated macrophages and that SOM plays an important role in stimulating NO production.
Figure 6.3: Effect of SN from C57BL/6 RPE eyecups on nitric oxide production by LPS-activated RAW cell macrophages and role of TGF-β and SOM.

24 hours after incubation of RPE eyecups with SFM, SN was collected and diluted 1 in 10. Experimental wells contained diluted SN of RPE eyecup together with LPS and RAW cell macrophages. In order to ascertain the role that TGF-β and SOM play, some experimental wells also received anti-TGF-β or anti-SOM antibodies. 2 µg/ml of recombinant SOM was added to some wells, with or without the addition of LPS, to confirm the role of SOM in controlling NO production. Wells containing RAW cells and LPS, acted as positive controls and those containing only RAW cells served as negative control. The cultures were incubated for 24 hours at 37°C.

Accumulation of nitrite in the culture supernatant of LPS-activated RAW cell line was assayed as an indicator of nitric oxide production. Supernatants of the wells were removed and assayed for NO production. Bars represent the mean ± SEM of triplicate wells from a representative experiment. * indicates mean values significantly greater than the positive control effect ($p<0.05$). ** indicates means significantly less than SN of RPE eyecup effect ($p<0.01$). *** indicates means significantly less than SN of RPE eyecup effect ($p<0.05$).
Figure 6.3

![Graph showing NO production (micromol/l) for different conditions.](image)

- **Positive control**
- **Negative control**
- SN + RPE eyecup + LPS + RAW cells
- RAW cells
- SN of RPE eyecup
6.2.4 Effect of SN of RPE eyecup on cytotoxicity of natural killer (NK) cells

Since aqueous humour was shown to suppress the ability of NK cells to lyse YAC-1 target cells in vitro (Apte 1996), it was of interest to see whether SN of RPE eyecups is similarly able to inhibit NK cell cytotoxicity. NK cell activity was augmented in vivo by injection of 100 μg per mouse of poly I: C intraperitoneally 24 hours prior to the harvesting of spleen, as previously described (Santoni 1985). Splenocytes rich in activated NK cells were harvested, and red blood cells were lysed. Splenocytes were co-cultured with \^{51}Cr labelled YAC-1 target cells in various effector to target ratios. After 4 hours, supernatants of these cultures were removed and the amount of \^{51}Cr release was measured. NK cells were fully capable of lysing YAC-1 cells in an effector: target ratio-dependent manner (Figure 6.4). SN of RPE eyecup significantly inhibited NK cell toxicity at all effector: target ratios tested (Figure 6.4). Addition of anti-TGF-β antibody reversed the SN induced inhibition implying that TGF-β plays a role in this process.
Figure 6.4: The ability of *in vivo* activated NK cells to lyse YAC-1 target cells and role of TGF-β

Diluted SN of RPE eyecups and poly I: C activated NK cells were incubated, at various effectors: target ratios, with $^{51}$Cr labelled YAC-1 cells for 4 hours at 37°C. Anti-TGF-β antibody was added to some wells to assess the role of TGF-β in NK cell cytotoxicity. As a control, activated NK cells were incubated without RPE SN at the same effector: target ratios. The spontaneous release of $^{51}$Cr from the target cells was determined by counting the supernatant from three wells containing only YAC-1 target cells and no effector cells. The total release was determined by measuring chromium released from the supernatant of 3 wells containing the YAC-1 cells and 1N HCl. Spontaneous release of chromium never exceeded 20% of the total release.
Figure 6.4

![Graph showing cytotoxicity data with different categories: SN of RPE eyecup, Positive Control, and anti-TGF-β Ab. The x-axis represents the ratio of Effector:Target (250 to 1, 100 to 1, 50 to 1), and the y-axis represents % Cytotoxicity ranging from 0 to 40.](image)

- SN of RPE eyecup
- Positive Control
- anti-TGF-β Ab

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6.3 Discussion

Immune privilege against the adaptive immune system in the subretinal space is well established. The results of the experiments reported here demonstrate that immune privilege against innate immunity also exists in the subretinal space. SN of RPE eyecups inhibited IL-1β production by casein and LPS-activated neutrophils as well as LPS-activated macrophages. TGF-β and SOM play important roles in the inhibition of IL-1β production by neutrophils but have little or no effect on IL-1β production by activated macrophages.

LPS can activate neutrophils and macrophages by binding the CD14 receptor on the surface of inflammatory cells. CD14 is a small glycoprotein that exists in a membrane bound (mCD14) form on the surfaces of monocytes, macrophages and neutrophils, and in a soluble form (sCD14) in plasma. LPS binding of CD14 mediates a variety of inflammatory responses, such as cytokine production by macrophages and neutrophils and adhesive responses of neutrophils (Hailman 1996, Schumann 1990, Wright 1991, Lee 1992) through Toll-like receptor 4 (TLR) 4. Cellular responses to LPS through mCD14 are augmented by LPS-binding protein (LBP), an acute phase plasma protein (Tobias 1986, Schumann 1990, Wright 1991, Lee 1992). In the serum free culture medium used in the present experiments, LPS binding protein was not present. Despite the lack of LBP, both macrophages and neutrophils were able to produce an appreciable amount of IL-1β in response to LPS demonstrating that they were sufficiently activated. Although presence of LBP is necessary for binding of low concentrations of LPS to mCD14, Hailman et al. demonstrated that sCD14 can form complexes with LPS in the absence of LBP and work with similar efficiency as when LBP is present.

Since RPE cells express CD14 on their surface and produce the soluble form of this receptor (sCD14) (Elner 2003), we propose that in serum free culture system, soluble sCD14 produced by RPE forms complexes with LPS, which in turn can activate RAW cells and neutrophils leading to cytokine production. However, the SN of RPE eyecups significantly inhibited IL-1β production by macrophages and neutrophils pointing to the presence of other factors in the SN that have the potential of inhibiting inflammatory responses produced by LPS via the CD14 receptor. TGF-β is reported to reduce expression of inflammatory cytokines by LPS-activated mouse macrophages through down-regulation of the CD14 receptor (Imai 2000) and this may be the mechanism through which IL-1β production by
LPS-activated neutrophils is inhibited. In this study TGF-β did not play a role in IL-1β production by activated macrophages as evidenced by the fact that blockade of TGF-β in the RPE SN did not reverse IL-1β inhibition. It is possible that the blockade of TGF-β was not sufficient or that different concentrations of TGF-β may yield different results. In their experiments, Imai et al. had pre-treated macrophages prior to the addition of LPS, while in this study, both LPS and TGF-β produced by RPE were present at the same time. RPE may also produce other factors that are capable of inhibiting IL-1β production by RAW cells. Experiments are underway to clarify these quandaries.

In contrast to IL-1β production, nitric oxide production by LPS-activated RAW cells was enhanced after addition of SN of RPE eyecup. LPS-induced effects on NO production versus that on IL-1β production in RAW cells seem to be differently regulated. There is evidence of differential regulation of LPS-induced responses in the literature. TNF-α was shown to be regulated via a CD14-dependent pathway at both low and high concentrations, while production of IL-1β and IL-1α was found to be regulated by two mechanisms depending on the presence or absence of plasma (Netea 1998). If plasma and presumably LBP are present the CD14-dependent pathway is undertaken and in the absence of plasma, a CD14-independent mechanism may take effect (Netea 1998). A CD14-independent pathway is also implicated in IL-6 production by CD14 double negative macrophages with high dose LPS. It may also be possible that LPS-CD14 complexes act through a different Toll receptor. The differential regulation observed may also be related to the dose of sCD14 present in the SN of RPE eyecup, which may act in such a way as to promote NO production while inhibiting IL-1β secretion.

Ocular cells are vulnerable to the cytotoxic effect of NO, as a pro-inflammatory molecules produced by macrophages (Streilein 2000) but there is evidence that NO may play a beneficial role in immune privilege of the subretinal space. RPE cells from rats co-cultured with activated lymphocytes produce high amounts of NO (Liversidge 1994), which may play a role in maintenance of immune privilege of the subretinal space by its cytotoxic effect on lymphocytes. Zech et al. demonstrated that NO elevated transepithelial electrical resistance (TER) helping the integrity of tight junctions among RPE cells that form the outer blood-retinal barrier (Zech 1998). So it is possible that the ability of RPE SN to potentiate NO production by macrophages is beneficial in maintaining the immune privilege of the
subretinal space. The present study demonstrated that SOM regulates NO production by macrophages but not IL-1β production. SOM has been reported to inhibit iNOS expression and NO production in peripheral macrophages (Kang 2001) and Kupffer cells (Chao 1999). Perez et al. recently demonstrated that there are 2 subtypes of SOM receptors on the surface of murine macrophages: SSRT1 with high affinity and low capacity, and SSTR2 with low affinity and high capacity (Perez 2003). One can speculate that macrophages may respond differently to SOM depending on the receptor through which SOM is acting. SOM inhibits IFN-γ production by T cells in schistosomiasis granulomas by acting through SSRT2 receptor (Elliott 1994). It is possible that SOM in this study potentiates NO production by acting through a different receptor, possibly SSRT1.

Similar to AqH, SN of RPE eyecups inhibited NK cell mediated lysis of YAC-1 target cells by a process that is, at least partially, TGF-β dependent. The ability of RPE cells to inhibit NK cell cytotoxicity is potentially very important (Apte 1996). RPE cells express low levels of class I MHC and can be a target for NK cells. During an inflammatory response NK cells may enter the subretinal space and cause destruction of RPE cells leading to disturbances of the outer BRB and diminishing immune privilege in the subretinal space. The neurons in the retina are devoid of class I expression and as such a potential target for and entry of NK cells through the blood retinal barriers may be devastating to the retina.

Factors other than TGF-β are also reported to be responsible for inhibition of NK cell cytotoxicity. Repp et al. reported that uveal melanomas produce macrophage migration inhibitory factor (MIF), which inhibited NK cell lysis of tumour cells (Repp 2000). Additionally Apte et al. demonstrated that AqH, partially through its content of MIF, inhibited NK cell killing of corneal endothelial cells (Apte 1997). MIF is produced by activated T cells, macrophages (Calandra 1994) and pituitary cells (Bernhagen 1993) and has an important role in delayed hypersensitivity (DH) (Bernhagen 1996). In the eye, mRNA and protein expression of MIF are reported in human corneal endothelial and epithelial cells, in human trabecular meshwork, and in iris/ciliary body epithelium (Matsuda 1996, Takase 2002). Matsuda et al. also discovered that Müller cells, retinal astrocytes and RPE are retinal sources for MIF production (Matsuda 1997). It is, therefore, possible that MIF is present in the SN of RPE eyecup and plays a role in inhibition of NK cell cytotoxicity.

Recently Hack et al. demonstrated a role of for serpin proteinase inhibitor (PI) 9-present in high protein concentration in immune privileged tissues including the eye- as an
important mediator of CTL/NK induced apoptosis of target cells. PI9 works by inhibiting the activity of granzyme B leading to prevention of degranulation in CTL and NK cells (Bladergroen 2001). There are no studies to show that PI9 exist in the retina or RPE. Pigment epithelium-derived growth factor (PEDF) produced by RPE, however, belongs to the serine proteinase inhibitor (serpin) family (Steele 1993) and may act similarly to PI9 in modulating NK/CTL cytotoxicity. Experiments to ascertain the role of PEDF in innate immune privilege of the subretinal space are underway.

It is now well established that innate immune privilege exists in the anterior chamber along with adaptive immune privilege. We believe that our data supports, for the first time, the proposal that innate immune privilege also exists in the subretinal space. The evidence suggests that soluble products of RPE contribute to inhibition of innate immunity in this compartment. It remains to be determined the extent to which adaptive immune privilege relies on the presence of innate immune privilege for its expression. Perhaps the recent observation of Sohn et al. is pertinent. In their experiments, iC3b, an activation product of the innate immune complement system, is required for ACAID induction, in part because it binds to CR3 on antigen presenting cells, and enables them to deliver ACAID-inducing signals to responding T cells (Sohn 2003). The result of experiments presented here makes one hopeful that immune privilege can be modulated through several mechanisms and this ability may be beneficial in order to restore immune privilege to eyes that have lost it due to disease.
Chapter 7 – General discussion and future directions

Advances in medicine and technology have given rise to hopes that transplantation may offer cure. Solid organ transplantation has moved from the realm of fantasy to the ordinary and corneal transplantation is the most successful transplantation of all. Retinal transplantation poses unique problems, as the neural tissues of the host and the transplanted material need to integrate, form functional units, and transmit correct information to the visual centres of the brain and the brainstem. Retinal transplants also face the formidable barrier of immune rejection. The subretinal space, where the transplants are placed, is an immune privilege space. This property is of fundamental importance if transplanted materials are to survive and become functional. Since retinal pigment epithelium lines the subretinal space in its entirety, and since RPE cells interact with the immune system in a complex way, the focus of this study was to investigate the role of RPE in protecting the subretinal space and retina from destructive inflammatory responses, such as those that can cause graft rejection.

By occupying a unique anatomical position between the retina and the choroid, RPE can regulate the passage of cells and their products between the two compartments: The choroid, where the immune cells exist in abundance, and the retina which is ordinarily devoid of immune cells and contains multiple evolutionary conserved autoantigens. This strategic positioning, together with the immunosuppressive activities of RPE demonstrated in the results of this dissertation, indicate that RPE is the master organiser of immune privilege in the subretinal space. Immune deviation, an essential part of immune privilege, is dependent on the integrity of the outer BRB, and on the ability of the RPE to produce active TGF-β. If either of these features is deficient, the immune deviation component of immune privilege in the subretinal space is degraded. The results also indicate that eyes with RPE that have been injured chemically, or that carry disrupted genes responsible for immunosuppressive factors, are deficient in their capacity to create an immunosuppressive microenvironment. Therefore, two important aspects of immune privilege in the subretinal space, immune deviation and the presence of immunosuppressive microenvironment, depend almost entirely on the ability of RPE to function normally.
In naming RPE the master organiser of immune privilege in the subretinal space, one must remember to consider other cells with potential immunosuppressive abilities in adjacent tissues. Müller cells traverse the retina and probably play a part in the formation of the inner BRB (Tout 1993). Caspi et al demonstrated that Müller cells profoundly inhibit proliferation of both antigen and IL-2 activated T cells in a non-MHC dependent manner (Caspi 1989). Similar to RPE, IFN-γ-treated Müller cells were found to secrete IL-1 and to be able to present Ag to a S-Ag specific T-helper cell line (Roberge 1988). Thus, Müller cells may also play an important part in immune regulation of the subretinal space, and their contribution needs to be studied further.

This study demonstrates for the first time that RPE cells are inherently resistant to immune destruction by allo-sensitised T cells in a non-FasL dependent manner. The ability of RPE to survive under attack leads to maintenance of a functional outer BRB and explains partially why allogeneic retinal grafts placed in the subretinal space survive for such an extended time before eventually succumbing to immune destruction. It is not clear how vulnerable RPE is to lysis by the cells and molecules of the innate immune system. Apte et al found that corneal endothelium, through its low expression of the MHC class I, is a target for NK-mediated lysis and that AqH prevented this action (Apte 1996). Based on a relatively low level of the MHC I antigen expression (Benson 1992b), RPE is also potentially vulnerable to NK-mediated attack, but there is no knowledge whether the subretinal space microenvironment is able to protect RPE cells from this type of destruction. Data presented in chapter 5 provides indirect evidence that RPE is resistant to destruction by innate immune cells. During the process of EAU in C57BL/6 mice, the RPE was only minimally damaged even though there must have been a large number of neutrophils, macrophages, NK and NKT cells in the subretinal space during the peak of the disease. In contrast, the RPE from TSP-1KO mice suffered severe damage. Using both in vitro eyecup model and in vivo model of injecting innate immune cells into the subretinal space, the vulnerability of RPE to the cells of innate immune system could be conveniently investigated in the future.

Soluble factors produced by the RPE were studied in our experiments. Active TGF-β2, SOM and TSP-1 were found to be amongst the most important factors in maintenance of adaptive immune privilege. TGF-β2 also seems to play an essential role in innate immune privilege by inhibiting NK cell toxicity and neutrophil activation. If one is to consider the environment of the subretinal space as comparable to that of the anterior chamber, one needs
to seek whether the factors present in the aqueous humour also exist in the interstitial fluid bathing the subretinal space. Since the RPE was shown for the first time in our experiments to produce SOM, a neuropeptide, the possibility exists that the RPE can produce other neuropeptides such as α-MSH or CGRP. These neuropeptides have been shown to have an important role in the immunosuppressive microenvironment of the anterior chamber and their production by the RPE should be investigated.

APCs treated with aqueous humour acquire the ability to induce ACAID when injected intravenously into a naïve animal. This induction takes place through TGF-β2 content of aqueous humour. Masli et al also demonstrated that addition of TSP alone to APCs is enough to confer on them ACAIDogenic properties, probably through activating TGF-β produced by the APCs themselves (Masli 2002). Since SN of the RPE eyecup contains TGF-β and TSP, it seems logical that they also have the ability to convert APCs into ACAID inducing ones. F4/80+ cells are identified as the macrophage population that conveys ACAID signals from the anterior segment into the spleen (Wilbanks 1991). In the subretinal space, however, it is not clear which cells are conveying ACAID signals to the spleen and indeed through which route they exit the subretinal space. Microglia may be the equivalent cells to F4/80+ macrophages of the anterior segment. Ma and Streilein demonstrated that microglia may act as “passenger leucocytes” (Ma 1998) and cultured microglia secrete significant amounts of mature TGF-β2 and smaller amounts of IL-12. Moreover, cultured microglia directed the responding T cells away from Th1 pathway and induced ACAID when injected into the eye of naïve allogeneic mice. It would be interesting to study other populations of APCs in the uvea and choroid to see if they are also ACAIDogenic alone or whether they would acquire this ability after treatment with SN of the RPE eyecup.

AqH was shown to have the ability to convert both naïve and primed T cells into regulatory T cells (Nishida 1999). Kitaichi and Taylor demonstrated that mice recovering from EAU acquire regulatory T cells in their spleen that once injected intravenously into naïve EAU-susceptible mice, can lead to the suppression of uveoretinitis (Kitaich 2003). They also demonstrated that production of these regulatory T cells is dependent on the presence of an immunosuppressive ocular microenvironment. Moreover, presence of these regulatory T cells prevents development of memory immune responses to autoantigens (Kitaichi 2003). Yoshida and colleagues cocultured naïve T cells with the iris/ciliary body pigment epithelial (I/CB PE) cells. T cells exposed to I/CB PE, through enhanced production
of TGF-β, inhibited anti-CD3 induced activation of bystander T cells and suppressed the expression of DH (Yoshida 2000). It appears that I/CB PE has the potential of producing regulatory T cells. Experiments determining if SN of the RPE eyecup is also capable of converting T cells into regulatory T cells would be of immense interest both in terms of understanding the mechanisms involved in maintenance of immune privilege in the subretinal space and in terms of therapeutic intervention.

The results presented, provide a certain level of optimism that the presumed “immunological barriers” to transplantation of allografts into the subretinal space may be low compared to other, non-ocular sites. However, this optimism is tempered by previous reports that the allogeneic RPE grafts placed subretinally can eventually be rejected (Jiang 1995). Caution is further warranted by the knowledge that diseases, especially those of an inflammatory and angiogenic nature, that disrupt the RPE, suprachoroidal space and the choriocapillaris, may disrupt immune privilege in this region. This point is well illustrated by the presence of severe, unrelenting and destructive uveitis in TSP-1 KO mice. Induction of uveitis in TSP-1KO as well as in wild type control mice is associated with influx of large number of immune cells and production of proinflammatory factors. Wild type C57BL/6, in contrast to TSP-1KO mice, suffered milder, shorter disease duration with limited retinal destruction. Presence of TSP, either through its ability to activate TGF-β or through its modulation of antigen presenting cells, or via other mechanisms as yet unknown, appears to be essential for maintenance of immune privilege mechanism in the subretinal space.

Immune privilege is regarded as an evolutionary adaptation that allows the delicate visual axis to be protected from destructive aspects of immunogenic inflammation while protecting the eye from pathogens. Streilein et al used presence of ACAID, survival of tumours in the anterior chamber, and absence of cells and proteins in aqueous humour as measures of persistence of ocular immune privilege during intraocular inflammation (Streilein 2002b). They concluded that “maintenance of immune privilege in the face of ongoing inflammation depends upon the emergence of progressive and partially different immunosuppressive mechanisms”. It appears that there are multiple nested mechanisms in the anterior segment, which can take over the task of protecting the visual axis, should the more immediate mechanisms fail.

The fact that absence of TSP led to inability of RPE to activate TGF-β and to loss of immune deviation in the subretinal space, points to the importance of this molecule in
maintaining immune privilege in the subretinal space. Perhaps these findings indicate a more limited array of nested mechanisms in the posterior part of the eye as compared to the anterior segment. One can speculate that the concept of a comparatively limited redundancy in the posterior part of the eye makes sense from an evolutionary standpoint. If the “window” of the eye (cornea and lens) becomes opaque due to inflammation or other disease processes, vision is lost. If a portion of the retina succumbs to destruction, the visual system can still function albeit with a reduced accuracy. This limited vision may be sufficient to make a difference between life and death in nature’s game of survival.

In this study, cell-to-cell interactions between the RPE and immune cells were not investigated. RPE produces a physical barrier to the entrance of leucocytes from the choroidal circulation and leucocytes have to gain entry into the subretinal space by passage through RPE cells (intracellular) as well as through the tight junctions (intercellular) between the cells. Moreover, RPE expresses CD36, a scavenger receptor, and can present antigen to T cells, albeit inadequately (Ryeom 1996). It is necessary to determine if and how this antigen presentation takes place and whether antigen presentation by RPE leads to production of tolerance in T cells, or promote destructive inflammation.

The evidence acquired in this study points not only to some of the mechanisms by which immune privilege in the subretinal space is regulated but also to measures that can be taken to restore immune privilege in this compartment, an important strategy for recovery from uveitis and in promoting the immunological success of allografts in the future.

Therapeutic implications of the findings in this study and those of Kitaichi et al can be immense both in terms of treatment for uveitis and prevention of allografts rejection in the subretinal space. In the animal model of EAU, spontaneous recurrence of uveoretinitis does not occur. The repetitiveness of the human disease, however, leads to cumulative destruction of the retina and uvea, and eventually to blindness. Kitaichi et al speculated that during an episode of EAU, the ocular microenvironment might be able to generate regulatory T cells leading to suppression and prevention of further uveitic episodes (Kitaichi 2003). Prevention of recurrence in human disease may be possible if regulatory T cells, formed by bathing them in the factors present in the immunosuppressive microenvironment of the subretinal space, are similarly administered to uveitis sufferers. It may also be feasible to induce tolerance to grafted material in the individuals receiving retinal transplantation, by first vaccinating the individuals with regulatory T cells produced against the alloantigens of the grafted material in
presence of immunosuppressive factors present in the subretinal space. Since it appears that
presence of a healthy and functional monolayer of RPE is essential in maintaining the
immune privilege of the subretinal space, strategies to replace diseased RPE in eyes requiring
retinal transplantation due to retinal diseases maybe beneficial before subsequent
administration of retinal/stem cells, to restore damaged neural retinas.
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Appendix

Serum free cell culture medium
RPMI 1640
0.1M HEPES
0.1% bovine serum albumin
1μg/ml iron-free transferring
10 ng/ml linoleic acid
0.2 ng/ml Na$_2$Se
0.2 μg/ml Fe(NO$_3$)$_3$

Complete RPMI medium
RPMI
0.1 M HEPES
0.05% 2ME
1% Glutamate
1% penicillin/streptomycin
10% foetal calf serum

Mink Lung cells culture medium
EMEM
0.1 M HEPES
0.05% 2-mercaptoethanol
1% penicillin/streptomycin
10% foetal calf serum

RPE culture medium
DMEM
0.1M HEPES
0.05% 2-mercaptoethanol
1% penicillin/streptomycin
20% foetal calf serum
2% Glutamate

**IL-1β ELISA coating buffer**

4.3 g NaHCO₃
5.3 g Na₂CO₃

Make up to 1 litre with distilled H₂O, pH 9.4

**IL-1β ELISA blocking solution**

8.0 g NaCl
1.42 g Na₂HPO₄
0.2 g KH₂PO₄
0.2 g KCl
5.0 g bovine serum albumin (fraction V)

Make up to 1 litre with distilled H₂O, pH 7.4

**IL-1β ELISA assay solution**

8.0 g NaCl
1.42 g Na₂HPO₄
0.2 g KH₂PO₄
0.2 g KCl
5.0 g bovine serum albumin (fraction V)
1 ml Tween 20

Make up to 1 litre with distilled H₂O, pH 7.4

**IL-1β ELISA wash buffer**

9.0 g NaCl
1 ml Tween 20
Make up to 1 litre with distilled H2O, pH 7.4

**IFN-γ ELISA wash buffer**

8.0 g NaCl
1.42 g Na₂HPO₄
0.2 g KH₂PO₄
0.2 g KCl
0.05% Tween 20
Make up to 1 litre with distilled H₂O, pH 7.4

**IFN-γ ELISA coating buffer**

0.05 M Carbonate/Bicarbonate, pH 9.6 (Premeasured pellets from Sigma)

**IFN-γ ELISA substrate solution**

50 mM sodium phosphate, pH 7.2
1.5 mM MgCl₂
Vulnerability of Allogeneic Retinal Pigment Epithelium to Immune T-Cell–Mediated Damage In Vivo and In Vitro

Parisa Zamiri, Qiang Zhang, and J. Wayne Streilein

Purpose. Because retinal pigment epithelium (RPE) constitutively expresses class I major histocompatibility complex (MHC) molecules, and CD95 ligand and secretes immunosuppressive factors, the vulnerability of these cells to attack by immune T cells is open to question. This study was conducted to determine the vulnerability of allogeneic RPE to damage by specifically sensitized T cells, both in vivo within the subretinal space, and in vitro.

Methods. BALB/c lymphocytes presensitized to C57BL/6 antigens were injected into the subretinal space of eyes of C57BL/6 and gld/gld mice, and the eyes were examined clinically and histologically. RPE eyecups were produced from mouse eyes by removing the anterior segment and neuronal retina, leaving an intact monolayer of RPE. Sensitized BALB/c lymphocytes were placed in the RPE eyecup and incubated for 4 hours. The RPE layer of the eyecups was assessed by confocal microscopy for viability, after staining with propidium iodide and acridine orange.

Results. Eyes that received T cells sensitized to C57BL/6 antigens displayed a circumscribed patch of persistent choroidal "whitening" clinically and a disrupted RPE cell layer histologically at the injection site at 5 days after injection. By 14 days, only RPE cells at the injection site were lost. RPE in eyecup preparations was relatively resistant in vitro to cytosis by sensitized T cells, whether the eyecups were obtained from CD95-deficient or wild-type mice.

Conclusions. RPE monolayers, both in vivo and in vitro, are relatively resistant to immune-mediated attack by specifically sensitized T cells. This relative lack of vulnerability is independent of the expression of CD95 ligand by target RPE cells and implies that immune barriers to acceptance of allogeneic RPE transplants may be less than if transplanted cells are from nonocular tissues. (Invest Ophthalmol Vis Sci. 2004;45:177-184) DOI:10.1167/iovs.03-0211

Retinal transplantation is viewed with hope as a solution to the loss of vision that results from irreversible damage to the neural retina and to the retinal pigment epithelium (RPE). Neural retina and RPE are vastly different types of tissues, and each confronts the experimentalist with a unique set of barriers to successful transplantation. Our laboratory has been particularly interested in determining the immunologic barriers to the successful transplantation of allogeneic retinal pigment epithelium into eyes of rodents. We and others have demonstrated that RPE as a tissue expresses class I alloantigens encoded within the murine major histocompatibility complex (MHC, H-2), and we presume, but have no direct evidence, that RPE also displays, on these class I molecules, the peptides derived from minor histocompatibility (minor H) antigens. Under normal circumstances, the RPE expresses no class II MHC molecules, although the expression of these molecules can be induced by exposure to IFN-γ. Thus, in principle, allografts of RPE and intact RPE should represent suitable targets for CD8+ T cells (TCs) of the cytotoxic type. At present, there is no direct information in the literature concerning the vulnerability of RPE to destruction by allospecific cytotoxic TCs.

RPE grafts, as single-cell suspensions of cultured cells and as intact sheets prepared from neonatal mouse eyes, have been implanted in the anterior chamber, the vitreous cavity, and even into the subretinal space of mouse eyes. Because all these intraocular compartments are immune privileged, interpretation of the fate of allografts of RPE is complicated by the contribution to graft survival made by the site itself. Wengel and Streilein investigated this question when they transplanted sheets of allogeneic neonatal RPE tissue beneath the capsule of the kidney—a non-immune-privileged site. They reported that allogeneic grafts of RPE survived as well as syngeneic RPE grafts beneath the kidney capsule. The transplants showed no gross or histologic evidence of rejection for at least 8 weeks after implantation. This finding offers formal proof that neonatal RPE tissue has the inherent property of immune privilege. These investigators also showed that the privileged status of RPE allografts could be aborted if the grafts failed to express CD95 ligand (CD95L). Thus, similar to the corneal endothelium and to the Sertoli cells of the testes, RPE functions as an immune-privileged tissue in part through the constitutive expression of CD95L.

At present, it is not technically feasible to transplant sheets of RPE orthotopically into the subretinal space of mouse eyes. For this reason, we have embarked on a series of studies to determine the vulnerability of RPE to immune destruction by cytotoxic TCs using strategies that do not involve orthotopic transplantation of the tissue. We injected into the subretinal space suspensions of allogeneic lymphocytes containing TCs primed for class I antigens expressed on recipient eyes, and we layered fully functional primed allogeneic TCs on intact monolayers of RPE in posterior eyecups in vitro. Our results reveal a marked resistance of RPE to lysis by primed TCs, a resistance that appears to be unrelated to the expression of CD95L.

Methods

Animals

Adult male BALB/c, C57BL/6, and C57BL/6 gld/gld (B6.gld) mice, aged 6 to 8 weeks were obtained from the animal facilities at the Schepens Eye Research Institute, Department of Ophthalmology, Harvard Medical School, 20 Staniford Street, Boston, MA 02114; waynes@vision.eri.harvard.edu.

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From the Schepens Eye Research Institute, Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts.

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Corresponding author: J. Wayne Streilein, Schepens Eye Research Institute, Department of Ophthalmology, Harvard Medical School, 20 Staniford Street, Boston, MA 02114; waynes@vision.eri.harvard.edu.
Eye Research Institute or from Taconic (Germantown, NY). Mice were kept in a common room of the vivarium. All experimental procedures conform to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Injections, inoculations, and clinical examinations were performed under anesthesia induced by intraperitoneal injection of ketamine (Ketalar; Parke Davis, Paramus, NJ) at 0.075 mg/g of body weight, and xylazine (Rompun; Phoenix Pharmaceutical, St. Joseph, MO) at 0.006 mg/g of body weight. Enucleation of the eyes and removal of spleen and lymph nodes were performed after the animals were killed by cervical dislocation. Five mice were used in each experimental group, and all experiments were repeated at least twice with similar results.

Preparation of Allosensitized Effector TCs

BALB/c mice were immunized in the flank by injecting 1 × 10^7 C57BL/6 splenocytes on day 0. They were killed 1 week later, and their spleens were removed and rendered into a single-cell suspension. Red blood cells were eliminated using red blood cell lysing solution. The splenocytes were cocultured at a ratio of 5:1 effector to stimulator with irradiated (2000 rad) naïve lymphocytes from C57BL/6 mice for 5 days in complete RPMI medium (RPMI plus HEPES, 2-mercaptoethanol [ME], glutamate, penicillin-streptomycin, and 10% fetal calf serum). After 5 days, the cells were washed three times in Hank's balanced salt solution (HBSS). The live cells were counted and used as BALB/c anti-C57BL/6 effector cells in subsequent experiments. As a control, C57BL/6 anti-BALB/c effectors were produced in a similar manner.

Assay for Cytotoxic Activity

BALB/c anti-C57BL/6 effector cells were tested for cell-mediated cytotoxicity in a standard 4-hour 51Cr-release assay. Either P815, a mastocyte cell line derived from DBA2 mice, or EL4 cells, a lymphoma cell line derived from the C57BL/6 strain, were used as target cells. In addition, cell mixtures containing BALB/c anti-C57BL/6 effector cells were tested for presence and functionality of natural killer (NK) cells by using the YAC-1 lymphoma cell line as targets. All target cells were labeled with 300 μCi Na235CrO4 (New England Nuclear, Boston, MA) per 3 × 10^6 cells for 2 hours in a water bath at 37°C. Thereafter, target cells were washed three times and placed in a microtiter plate at a concentration of 2 × 10^4 per well. Either the effector BALB/c anti-C57BL/6 effector cells or C57BL/6 anti-BALB/c effector cells were added to the wells of the microtiter plate at the following effector-to-target cell ratios (E:T): 100:1, 10:1, 2:1, and 1:1. Culture plates were centrifuged at 1000 rpm for 5 minutes and incubated for 4 hours at 37°C. After incubation, 25 μL of the supernatant was removed and counted for radioactivity. The percentage of specific chromium release was calculated using the standard formula. The spontaneous release of 51Cr from the target cells was determined by counting the supernatant from three wells containing only target cells and no effector cells. The total release was determined by measuring chromium released from the supernatant of three wells containing the target cells and 1N HCl. Spontaneous release of chromium never exceeded 20% of the total release.

Injection into Subretinal Space

Subretinal space injections were performed according to the procedure of Whiteley et al.10 on anesthetized animals that, in addition, received topical proparacaine to anesthetize the ocular surface and tropicamide 1% to dilate the right pupil. Injection into the subretinal space was performed using very fine, bevelled, pulled-glass micropipettes that were connected to a 10-μL syringe (Hamilton, Reno, NV) by a fine polyethylene tube. The entire apparatus was filled with HBSS, but an airlock was produced before the volume to be injected, thereby preventing dilution of the injected material. The bore of the glass needle was coated (Sigmacare; Sigma-Aldrich, St. Louis, MO) to prevent adherence of cells. The injections were made under direct visualization by transscleral approach through the peripheral retina, using a binocular surgical microscope and a coverslip held on the cornea. The glass needle was advanced carefully until it reached the subretinal space where cells in a volume of 1 μL were injected.

Histologic Examinations

Mice were killed by cervical dislocation at various time intervals, and their eyes were enucleated and fixed immediately in 4% paraformaldehyde and embedded in methacrylate. Five-micrometer sections were cut and stained with hematoxylin and eosin. Tissue sections were then examined by light microscopy.

Preparation of Posterior Eyecups

Eyes were enucleated from C57BL/6 and B6.gld mice and placed in Ca2+/Mg2+-free HBSS on ice for 30 minutes, and then removed, and the muscles, connective tissue, and conjunctiva were excised with microrackets. A circumferential incision was performed below the level of the ciliary body, and the entirety of the anterior segment, including the cornea, iris, ciliary body, and the lens, which were discarded. The remaining tissue (posterior eyecup) was placed in 0.01 U/mL of chondroitinase ABC11 for 30 minutes at 37°C, placed on ice, and washed three times in HBSS. The neural retina was gently lifted off the RPE layer by microsurgical forces. Posterior eyecups consisting of sclera, choroid, and a healthy, intact monolayer of RPE were placed in individual wells of microculture plates (5 plate; Nunc Brand Products, Nalge Nunc International Corp., Naperville, IL) for further experiments.

In Vitro Cytotoxicity Assay

Posterior eyecups from eyes of C57BL/6 and B6.gld mice were placed in microwell plates (5 plate) and 10,000 effector BALB/c anti-C57BL/6 TCs in 10 μL of DMEM (fortified with glutamate, antibiotics, 2-ME, and 1% HEPES) were gently layered onto the eyecups. After incubation for 4 hours at 37°C, the eyecups were washed twice and stained with 1 μL/mL propidium iodide (PI) and 0.5 μL/mL acridine orange (AO) for 15 minutes. The cups were mounted on glass slides, covered with glass coverslips, and observed by confocal microscopy. For some experiments, the eyecups were incubated with 100 U/mL recombinant IFN-γ (BD Biosciences, San Diego, CA) for 4 or 12 hours before exposure to effector BALB/c anti-splenocytes. To assess the level of class I MHC, some eyecups were stained with FITC-conjugated anti-MHC class I antibody (R&D Systems, Minneapolis, MN).

RESULTS

Resistance of RPE Cells to Immune Destruction by Alloreactive Effector T Cells Placed Subretinally

Primed H-2b-specific cytotoxic TCs were generated by immunizing BALB/c mice subcutaneously with C57BL/6 spleen cells. One week later, spleen cells were harvested from these mice and restimulated in vitro with x-irradiated C57BL/6 spleen cells. In vivo primed TCs restimulated in vitro in this manner contain cytotoxic TCs specific for MHC and minor histocompatibility (minor H) antigens. After 5 days, the responding lymphocytes were harvested and assayed for their capacity to lyse EL-4 cells, a target tumor cell line derived from the C57BL/6 mouse strain. Control effector cells were prepared similarly by immunizing C57BL/6 mice with BALB/c spleen cells. As revealed in Figure 1A, BALB/c effector cells lysed target EL4 cells expressing H-2b class I and minor H alloantigens. EL4 cells were not lysed by C57BL/6 anti-BALB/c effector cells, demonstrating that the cytotoxic attack was specifically directed at C57BL/6 alloantigens. To demonstrate that C57BL/6 effector (C57BL/6 anti-BALB/c) cells had the ability to lyse BALB/c targets allospecifically, C57BL/6 effector cells were used in a cytotoxicity assay with P815 mast cytoma cells from DBA2 mice. Even though these target cells differ from BALB/c
at numerous minor H antigens, they share the same MHC antigens. Figure 1B demonstrates that C57BL/6 effector cells lysed target P815 cells expressing H-2d class I alloantigens. However, P815 cells were not lysed by BALB/c anti-C57BL/6 effector cells, demonstrating that both effector cell types were capable of allospecific cytotoxic killing. Because activated NK cells may also be present in the preparations of effector TCs generated, the ability of NK cells to lyse target cells was tested by using YAC-1 cells as targets. Figure 1A demonstrates that very limited NK cell killing was detected, even at high effector-to-target ratios.

Effector cells prepared in this manner were injected (10,000 cells per 1 μL inoculum) into the subretinal spaces of eyes of normal C57BL/6 mice. As the control, eyes of some mice received a 1 μL inoculum of PBS alone into the subretinal space. In one set of recipients, the eyes were examined clinically using fundoscopy at 2, 5, and 14 days after injection. In another set, injected eyes were removed at 1, 6, 24, and 48 hours and at 5 days and 14 days after injection. These eyes were prepared and sectioned for histologic examination.

Clinical examination after 2 days revealed that the retina was edematous in an area of one disc diameter (diameter of the optic nerve head), at the site of injection. This edematous appearance was detected irrespective of the contents of the subretinal injection. At the same time, the anterior chamber was normal, the lens was clear, and there was no evidence of intraocular inflammation. These findings indicate that there was a minimal amount of trauma associated with subretinal injections of this type and that the focus of this response is restricted to the site of injection. At 5 days after injection, in eyes that received BALB/c anti-C57BL/6 lymphoid cells subretinally, a distinct pallor developed at the injection site that was readily distinguishable from surrounding tissue and was approximately two disc diameters in size. This defect in the RPE layer was still present when these eyes were examined at 14 days after injection, but had not changed in size during this time interval. Of particular importance, no area of pallor was detected in the retinas of eyes that received injections of control lymphoid cells. Thus, injection of specifically sensitized lymphoid cells containing TCs created a circumscribed defect in the RPE monolayer at the site of injection. Once established, this lesion persisted through time but failed to expand in size.

Histologic examination of eyes receiving effector cell injections supported these observations (Figs. 2, 3, 4, and 5). In eyes that received effector cell injections, lymphoid cells were detected in the subretinal space at 1 hour (Figs. 2A, 2B). Among eyes that received control cells, no lymphoid cells were seen thereafter, but among eyes that received BALB/c anti-C57BL/6 effector cells, inflammatory cells persisted at the injection site through 24 hours after injection (Fig. 2C).

Figure 1. Ability of effector BALB/c anti-C57BL/6 or C57BL/6 anti-BALB/c cytotoxic TCs to lyse allospecific target cells. (A) BALB/c spleen cells primed against C57BL/6 targets were incubated with [3H]Cr-labeled EL4 cells, a C57BL/6-origin lymphoma cell line, for 4 hours at 37°C. As a control, C57BL/6 splenocytes primed against BALB/c targets were similarly incubated with EL4 cells. In addition, lymphoid cell mixtures containing BALB/c effector cells were incubated with YAC-1 lymphoma cells. (B) C57BL/6 spleen cells primed against BALB/c targets were incubated with [3H]Cr-labeled P815 cells, a DBA2 origin mastocytoma cell line, for 4 hours at 37°C. As a control, BALB/c spleen cells primed against C57BL/6 targets were similarly incubated with P815 cells.

Figure 2. Histologic appearance of subretinal space injection site of effector lymphocytes in C57BL/6 eyes. BALB/c anti-C57BL/6 effector cells (n = 10,000) were injected into the subretinal space of adult C57BL/6 mice. One hour and 24 hours later, the animals were killed and the eyes were enucleated, placed in 4% paraformaldehyde, embedded in methacrylate, sectioned, stained with hematoxillin and eosin, and viewed by light microscopy. (A) Injection site and the bleb produced around the site of injection. (B) Magnified view of the injection site shows numerous lymphocytes. A similar image was observed when C57BL/6 anti-BALB/c spleenocytes were injected into the subretinal space of C57BL/6 mice. (C) Section from an eye receiving BALB/c anti-C57BL/6 spleenocytes 24 hours after injection. Arrow: injection site. A retinal detachment was evident throughout the length of the retina, yet few lymphoid cells were dispersed through the subretinal space. None of the injected cells were found at 24 hours in other control experiments.
Figure 3. Histologic appearance of RPE and retina around the site of injection of C57BL/6 effector lymphocytes (C57BL/6 anti-BALB/c) in C57BL/6 eyes. (A) Control C57BL/6 effector cells sensitized to BALB/c alloantigen were injected into the subretinal space of adult C57BL/6 mice. Two weeks later, the eyes were enucleated and prepared for histology. The RPE layer was intact and the retina had normal cytostructure. (B) High magnification showing the RPE cell nuclei. No inflammatory cells are visible.

At 48 hours after injection, all eyes contained edema-induced fluid in the subretinal space and within the retina itself around the site of injection. When examined at later intervals, neither the sham-injection eyes (data not shown) nor eyes that had received C57BL/6 anti-BALB/c TCs demonstrated any edema or inflammatory cells, and the RPE layer was intact (Fig. 3). By contrast, at 5 days the RPE layer was disrupted at the site of injection of BALB/c anti-C57BL/6 cells. Some RPE cells appeared vacuolated and had lifted off Bruch's membrane (Fig. 4A). At 14 days after injection, the injection site was devoid of healthy RPE cells, and there was a sharp perimeter to the lesion, formed by healthy-appearing RPE (Figs. 4B, 4C). At no time were inflammatory cells observed within the neural retina, nor was the photoreceptor layer of the retina damaged.

Together, the results of these experiments indicate that specifically sensitized effector lymphoid cells containing TCs can attack and destroy RPE at the precise site where they are injected but that the damage remains confined to this site, with little evidence to suggest that the effector cells can systematically and sequentially destroy adjacent RPE cells.

Resistance of CD95 Ligand Deficient RPE Cells to Immune Destruction by Effector T Cells Placed Subretinally

Because RPE express CD95L constitutively and because CD95L expression on layers of allogeneic neonatal RPE implanted beneath the kidney capsule protects these grafts from immune destruction, we next examined whether enhanced destruction of RPE might occur when specifically sensitized lymphoid cells are injected into the subretinal space of B6.gld mice that do not express a functional CD95L molecule. C57BL/6-primed lymphoid cells restimulated in vitro were injected into the subretinal space of B6.gld mice, and the injected eyes were examined clinically and histologically as described earlier. As revealed in Figure 5, the pattern of clinical and histologic findings in CD95L-deficient eyes that received injections of both specifically and irrelevantly sensitized lymphoid cells was identical with that found in normal eyes. In particular, the initial RPE lesion, its evolution, and final extent were no different in eyes of CD95L-deficient mice than in those of wild-
The results of the experiments described to this point suggest that the constitutive level of MHC class I expression is insufficient to trigger effector cells. To address this point, class I expression was stimulated in posterior eyecups by exposing the tissues in vitro to IFN-γ for 4 or 12 hours, before testing the effect of specifically sensitized T cells on RPE cell lysis. Figure 7A reveals that the RPE cells in the eyecup preparation, without treatment with IFN-γ, expressed low levels of class I MHC antigens. There was a slight upregulation of class I MHC after the RPE eyecups were treated with IFN-γ for 4 hours (data not shown), but, after treatment with IFN-γ for 12 hours, class I MHC was significantly upregulated (Fig. 7B). We determined that treatment of the RPE eyecup with IFN-γ for 12 hours without the addition of BALB/c effector TCs did not induce toxicity in the RPE layer on its own (data not shown). Prior treatment of the RPE eyecups with IFN-γ for 12 hours led to a higher uptake of PI on exposure to TCs than did untreated RPE (Fig. 6D). A comparatively small, specific RPE cell death was detected in eyecups treated with IFN-γ for only 4 hours (Fig. 6C). These results suggest that low-level expression of MHC class I molecules on RPE eyecups from normal eyes is at least one factor that renders RPE cells relatively resistant to lysis by allospecific TCs.

Relative Resistance of RPE to T-Cell-Mediated Damage

One possible explanation for the relative invulnerability of RPE in the posterior eyecups to lysis by TCs is that the constitutive level of MHC class I expression is insufficient to trigger effector cells. To address this point, class I expression was stimulated in posterior eyecups by exposing the tissues in vitro to IFN-γ for 4 or 12 hours, before testing the effect of specifically sensitized T cells on RPE cell lysis. Figure 7A reveals that the RPE cells in the eyecup preparation, without treatment with IFN-γ, expressed low levels of class I MHC antigens. There was a slight upregulation of class I MHC after the RPE eyecups were treated with IFN-γ for 4 hours (data not shown), but, after treatment with IFN-γ for 12 hours, class I MHC was significantly upregulated (Fig. 7B). We determined that treatment of the RPE eyecup with IFN-γ for 12 hours without the addition of BALB/c effector TCs did not induce toxicity in the RPE layer on its own (data not shown). Prior treatment of the RPE eyecups with IFN-γ for 12 hours led to a higher uptake of PI on exposure to TCs than did untreated RPE (Fig. 6D). A comparatively small, specific RPE cell death was detected in eyecups treated with IFN-γ for only 4 hours (Fig. 6C). These results suggest that low-level expression of MHC class I molecules on RPE eyecups from normal eyes is at least one factor that renders RPE cells relatively resistant to lysis by allospecific TCs.
There are blinding diseases of the eye in which dysfunction and loss of RPE is thought to be the primary pathogenic condition. Leber’s amaurosis is genetically determined disease in which mutation of a protein uniquely expressed in RPE renders these cells incapable of supporting photoreceptors, and eventually the retina deteriorates. In age-related macular degeneration, a multifactorial disease of unknown cause, early changes and loss of RPE are thought to be central to the pathogenesis. For these and similar diseases, transplantation of allogeneic RPE to replace defective or depleted RPE is an attractive potential clinical solution. Allogeneic tissues as grafts are vulnerable to immune rejection, and this potential exists in principle for allogeneic RPE grafts. The importance of this potential is mitigated, on the one hand, by the immune-privileged nature of the subretinal space in which RPE grafts are to be placed. Allogeneic tissues implanted into this site have extended survival, and alloantigenic material and soluble protein antigens injected into the subretinal space induce a form of immune deviation very similar to that induced by injection of antigens into the anterior chamber. On the other hand, there is unequivocal evidence that RPE tissue has properties of an immune-privileged tissue. RPE cells express transplantation antigens that should make them vulnerable to immune recognition, and there is evidence that allografts of RPE placed intraocularly, even in the subretinal space, are subject to destruction that is presumed to be immunologic. The experiments reported herein explored this possibility.

Our results indicate that explanted, intact layers of RPE resident in posterior eyecups are quite resistant to lysis by TCs that are fully able to lyse other nonocular types of target cells. Moreover this relative resistance to TC-mediated lysis displayed in vitro by RPE in posterior eyecups was mirrored to some extent when specifically sensitized TCs were injected into the subretinal space of appropriate recipients. The positive result is that effector TCs created small, circumscribed lesions at the site of injection, and eventually these sites were found to be depleted of RPE. Moreover, the constraint applied to the injected cells was not provided by CD95L. Lesions produced in the RPE layer of B6.gld eyes were similar in all respects to those produced in wild-type C57BL/6 eyes.

The relative resistance of RPE to lysis by TCs may be due to mechanisms inherent within RPE cells, such as their low level of class I MHC expression, or the ability of the RPE to resist the lytic mechanisms of activated TCs. RPE cells are known to undergo apoptosis as a result of several stress factors, such as hydrogen peroxide (H$_2$O$_2$), ischemia, and blue-light toxicity. RPE, however, has multiple mechanisms to limit the apoptotic process. Alge et al. have demonstrated that RPE cells produce a heat shock protein, α-crystallin, that limits apoptosis due to oxidative stress. Others have noted that pigment epithelium-derived factor (PEDF) produced by RPE cells inhibited apoptosis in cultured retinal neurons. Presumably, PEDF works similarly to inhibit RPE cell apoptosis from hydrogen peroxide injury. Overexpression of Bcl-2 has been shown to decrease apoptosis in human RPE exposed to H$_2$O$_2$ or blue light.

TCs exert their lytic effects, either by synthesis and secretion of cytokines such as IFN-γ or TNF-α, or by direct cell-to-cell contact. There are two distinct, contact-dependent mechanisms that are used by TCs to lyse their targets: (1) perforin-granzyme-mediated cytotoxicity, with directional release of granular content from TC cytoplasm toward the target cells, and (2) a receptor-mediated cytotoxicity involving interaction of FasL or TNF-α on the RPE cells with Fas or TNF-α receptors on the target cells. The final common pathway is the activation of caspases leading to apoptotic cell death. Perforin-mediated apoptosis in the retina has been noted in relation to viral retinitis where the apoptotic cells are found mostly in the outer nuclear layer; however, it is not clear whether RPE cells undergo apoptosis by this pathway.

Subthreshold expression of MHC class I molecules seems to explain partially the resistance of explanted RPE to TC-mediated lysis. When posterior eyecups were treated for 12 hours with IFN-γ, class I expression on the RPE was enhanced, and specific TCs killed a modest, but significant, proportion of RPE cells treated in this manner. Low-level constitutive expression of MHC class I molecules is a characteristic feature of other ocular cells as well. Corneal endothelial cells are especially depauperate in class I expression, and this is believed to explain why donor-specific cytotoxic TCs induced in mice bear MHC-disparate orthotopic corneal allografts play no important role in acute graft rejection.

RPE also express Fas antigen and can be a target for apoptosis by FasL-bearing cells. RPE eyecups from C57BL/6 mice that were treated with allosensitized effector TCs did not show
any evidence of apoptosis, but once the eyecups were treated with IFN-γ for 12 hours, RPE cell lysis occurred. IFN-γ is known to increase Fas receptor expression in tumor cells, leading to increased apoptosis in these cells. M Multabacher et al. 27 demonstrated that TCs can indeed upregulate Fas expression as a result of IFN-γ release, but do not increase apoptosis in Fas-positive target cells in the absence of TC receptor (TCR) ligation. Upregulation of Fas receptor and increased Fas-FasL interaction may be one of the mechanisms by which RPE cells undergo apoptosis. An increase in IFN-γ levels accompanies injection of TCs into the subretinal space, in part due to production of IFN-γ by the injected cells. The expectation, therefore, would be for upregulation of class I MHC and Fas receptors throughout the subretinal space, leading to widespread lysis of the RPE. Instead, the lysis of the RPE remained quite localized in our experiments. What mitigates this positive result is our observation that these lesions failed to progress and expand through time. When TCs are added to nonocular target cells in vitro, they are able through time to eliminate every target cell. Yet, TCs placed in the subretinal space failed to display this capacity. It should be pointed out that the lymphoid cell suspensions injected into the subretinal space in these experiments contained primed allospecific CD4+ TCs, as well as CD8+ TCs. This is important, because histologic examination of injected eyes revealed almost no nonspecific inflammation at the injection site. We interpret these results to mean that RPE themselves secrete or the subretinal space in which the cellular inocula were placed contains factors that silence the destructive potential of effector TCs. Determining the nature and mode of action of these silencing factors is a major goal of our future experiments.

One of the strategies that may be used by RPE cells to avoid being lysed by TCs is to induce apoptosis in the activated TCs. RPE has been shown to induce apoptosis in activated TCs in both a cell-to-cell contact-dependent and a contact-independent manner. Jorgensen et al. 55 reported that cultured fetal human RPE cells (HFtRPE) incubated with an anti-CD3-activated TC population led to apoptosis of TCs in a Fas-FasL-dependent manner. In our experiment, the resistance to lysis was found to be unrelated to expression of CD95L, because TCs layered onto RPE of posterior eyecups prepared from CD95L-deficient mice also did not cause lysis. To our knowledge, this is the first report of an in vitro experiment designed to test whether constitutive CD95L expression protects ocular target cells from TC-mediated lysis. Our results provide a certain level of optimism that the presumed immunologic barriers to RPE transplantation may be low compared with other, nonocular solid tissues. Immune privilege of the subretinal space and of the RPE seems to be important. However, this optimism is tempered by previous reports that allogeneic RPE grafts placed subretinally can eventually be rejected. Caution is further warranted by the speculation that diseases, especially those of an inflammatory and angiogenic nature, that disrupt the RPE, suprachoroidal space, and the choriocapillaris, may very well disrupt immune privilege in this region. Restoration of immune privilege may be an important strategy for promoting the immunologic success of RPE grafts in the future.

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