Charles University in Prague
1st Medical Faculty

THE STUDY OF THE ROLE OF CELL POPULATIONS IN REJECTION AND TOLERANCE OF ORTHOTOPIC CORNEAL TRANSPLANTS

–

(IMMUNOMODULATORY APPROACHES IN CORNEAL ALLO- AND XENOGRAFT REJECTION IN MOUSE AND RAT)

BY

MUDR. KLÁRA SEDLÁKOVÁ

A thesis submitted in partial fulfillment of
the requirements for the degree
of Doctor of Philosophy

Study program: Cell biology and pathology

Prague, Czech Republic
2007

Declaration

Here, I declare that this thesis has been composed by me and has not been submitted in any previous application for a degree. I have carried out the reported work, except where acknowledged, and sources of information have been specifically recognized by means of references.

MUDr. Klára Sedláková

Abstract

Small animal models of orthotopic corneal transplantation offer many advantages for the study of immune mechanisms after grafting - not only because of the similar mechanisms of murine and human corneal transplant rejection but also due to the feasibility of the direct observation of the animal without the need to sacrifice it. The purpose of the thesis was to study this model in mouse and rat. We established allotransplantation (BALB/c to C57BL/6 mouse and Wistar Furth to Lewis rat) and concordant xenotransplantation model (rat to mouse; Lewis to BALB/c and Sprague Dawley to BALB/c) and set up grading schemes for the evaluation of the clinical course after grafting.

Initially, we focused on the effect of the suturing technique on the survival of xenografts and on the efficacy of selected immunosuppressants: cyclosporine A, monoclonal antibody against T cells (anti-Thy-1.2) and AMT (a specific inhibitor of inducible nitric oxide synthase, 2-amino-5.6-dihydro-6-methyl-4-H-1.3-thiazine)). The results demonstrate that the suturing technique significantly affects the outcome of transplantation and, importantly, influences the effectiveness of immunosuppressive regimens and therefore must be taken into account when evaluating the efficacy of immunosuppressive drugs.

FTY720 is a novel immunosuppressant with a completely new mechanism of action. It modifies patterns of T cell migration and sequestration in lymph nodes and the thymus. Our results show that treatment with FTY720, even in monotherapy, substantially delays the inflammatory response in a dose dependent manner after corneal concordant xenotransplantation and prevents the necrosis at the graft margin and sloughing of the xenograft, possibly enabling later graft infiltration. However, we

have also found that treatment with FTY720 induces a profound reduction in T and B cell expansion and the expression of B cell activation markers (major histocompatibility complex (MHC) class II and CD86) in draining lymph nodes (DLN) with subsequent impairment of late recruitment of inflammatory cells into the graft. We demonstrate that FTY720, used in isolation, is a potent immunosuppressant in the control of xenogenic corneal graft rejection and shows that it may be possible, at least in experimental settings, to develop long-term acceptance of corneal xenografts.

We also demonstrate that FTY720 is effective in corneal allograft rejection with a potential to reverse rejection even after priming. It limits the early corneal infiltration with CD11c+ cells and prevents both T and B cell expansion in the DLN with subsequent impairment of the late intragraft recruitment of inflammatory cells. FTY720 also selectively decreases cellularity in the DLN of grafted mice on day 20 after transplantation, suggesting that alloantigen-activated dividing cells can be preferentially affected by the FTY720-treatment.

We show that the reduction of MHC class II expression on lymph node B cells can be induced by FTY720 *in vivo* as well as *in vitro*. However, this effect is limited only to lymph node B cells, since CD11c+ or B220+ cells from the spleen are not affected. Although FTY720 restricts MHC class II expression on lymph node B cells, additional stimulation with lipopolysaccharide is capable of partially restoring this expression and thus another pathway involved in MHC class II regulation can to some extent operate in the presence of FTY720. In conclusion, FTY720 reduces levels of MHC class II expression on the surface of lymph node B220+ cells, which may represent an additional mechanism of FTY720-induced immunosuppression.

Corneal graft rejection is mediated mainly by donor-specific CD4+ T cells and the T_H1 response predominates. Redirecting the recipient's immune response from T_H1 towards T_H2 may have a positive effect on the corneal graft outcome. Transduction with AdrIL- 4 (alone or in combination with vIL-10) leads to visible attenuation of the iris vessels reaction shortly after the transplantation as well as during rejection. This happens in contrast to control mice or mice with grafts transduced with Adβ-gal or AdvIL-10 alone. Presumably, this reflects an immunosuppressive effect of IL-4 on the inflammatory reaction in the anterior chamber. *Ex vivo* corneal transduction with AdrIL-4 does not delay corneal graft rejection and, in addition, its application is dose dependently associated with increased corneal opacity. This probably occurs because of eosinophil infiltration induced by eotaxin produced by corneal fibroblasts under the influence of IL-4. Combined treatment of IL-4 and vIL-10 is associated with more pronounced corneal opacity, the increased activity of neovascularization; the combination of IL-4 with low titre of vIL-10 shortens the graft survival. To sum up and conclude, the redirection of the local immune response towards T_H2-type does not suffice to delay corneal allograft rejection. Nevertheless, the signs of immune modulations warrant further research.

Using small animal models in studies of corneal transplantation significantly extends our possibilities to understand immune mechanisms (and not only those associated with transplantation) and also assists in our efforts to uncover the efficacy and possible mechanisms of action of immunomodulatory drugs or approaches. This thesis demonstrates various possibilities these applications provide.

List of abbreviations

α-MSH - alpha-melanocyte-stimulating hormone

AC - anterior chamber

ACAID - anterior chamber-associated immune deviation

AMT - 2-amino-5.6-dihydro-6-methyl-4-H-1.3-thiazine, a specific inhibitor of inducible nitric oxide synthase

AP-1 - activator protein-1

APC - antigen presenting cells

BCR - B cell receptor

bFGF - basic fibroblast growth factor

CCTS - Collaborative Corneal Transplantation Studies

CGRP - calcitonin gene-related peptide

CsA - cyclosporine A

CSLN – cervical superficial lymph node

CTL - cytotoxic T cell

CTLA-4 - cytotoxic T-lymphocyte associated antigen 4

DAF - decay-accelerating factor

DC - dendritic cells

DLN - draining lymph nodes

DTH - delayed-type hypersensitivity

EBV – Ebstain-Barr virus

EDG - endothelial differentiation gene

F7D5 - monoclonal antibody against T cells anti-Thy-1.2

FasL - Fas ligand

FK-BP - FK-binding proteins

GVHD - graft versus host disease

HD – high dose

HLA - human leukocyte antigens

ICAM-1 - intercellular adhesion molecule–1

IFN-γ - interferon-gamma

IMPDH - inosine monophosphate dehydrogenase

iNOS - inducible form of nitric oxide synthase

KO - knock out

LC - Langerhans cells

LD – low dose

LFA-1 - leukocyte function-associated antigen-1

LN – lymph node

LPS – lipopolysaccharide

MCP - membrane cofactor protein

mHA - minor histocompatibility antigens

MHC - major histocompatibility complex

MIF- migration inhibitory factor

MIP-2 - macrophage inflammatory protein-2

MMF - mycophenolate mofetyl

mTor - mammalian target of rapamycin

NFκB - nuclear factor kappa B

NF-AT - nuclear factor of activated T cells

NK - natural killer cells

NKT- natural killer T cells

NO - nitric oxide

PKC - protein kinase C

RAD - rapamycin

RANTES (CCL5) - regulated upon activation, normal T cell expressed, and secreted

S1P - sphingosine-1 phosphate

S1P-R - sphingosine 1-phosphate receptors

SMLN – submandibular lymph node

SphK - sphingosine kinases

STAT - signal transducers and activators of transcription

TCR - T cell receptor

TGF-β - transforming growth factor-beta

TNF-α - tumor necrosis factor-alpha

TRAIL - tumor necrosis factor-related apoptosis-inducing ligand

VEGF - vascular endothelial growth factor

vIL-10 - EBV-encoded IL-10

VIP - vasoactive intestinal peptide

Table of Contents

Preface

Corneal transplantation and related immune mechanisms are studied at our department for a long time. Here, their history starts in the first half of 20th century and is connected with Professor Anton Elschnig and, later, Professor Jaromir Kurz. A further important advancement occurred with the introduction of experimental models of corneal transplantation in rabbit and later on in mouse when the group led by Professor Martin Filipec and Associated Professor Vladimír Holáň (from the Institute of Molecular Genetics of Academy of Sciences of the Czech Republic) was the only group in former Czechoslovakia focusing on this experimental topic.

In 1985, Williams *et al.* introduced a model of orthotopic corneal transplantation in rat (1) and soon afterwards, in 1990, She *et al.* in mouse (2). Six years later, Hašková *et al.* published their work on the role of major and minor histocompatibility antigens in mouse (3). We obtained a model that is – in comparison with rabbits – more defined, offers inbred and congenic strains and various immunologic reagents and is also less expensive. On the other hand, rat but especially mouse models are technically highly demanding and very challenging.

At the beginning of my Ph.D. studies, the knowledge of the model of corneal transplantation and related cellular and molecular mechanism was very fragmentary. In the following years, as the global awareness of the subject-matter among those who were involved in its studies world-wide gradually increased, the aims and the course of my work on the presented thesis had to be adjusted so that my research could reasonably compete with foreign research teams.

The general aim of the thesis was to study an experimental model of corneal transplantation in mouse and rat and to analyze cell populations involved. For this purpose, we used the methods of cellular and molecular immunology such as immunohistology, cytology or flow cytometry. The starting point was to establish experimental model of corneal transplantation in mouse and rat and grading schemes for the evaluation of the clinical course after grafting both in allo- and concordant xenotransplantation models.

The fact that the general knowledge of the subject substantially increased on a worldwide scale motivated the extension of the focus of the thesis above and beyond the outlined general aim. This extension was, however, always directed to be closely linked to the main topic of the thesis. Using several new immunomodulatory approaches offered new opportunities in this regard. In addition, the possibility to participate in the work of various foreign research teams (under the leadership of Prof. Forrester, Prof. Pleyer and Dr. Ritter) enriched the scope of the research, as well as the methodology employed.

The organization and the structure of the thesis reflect the fact that its subject was not studied at a single institution. The conditions at each of the research centers have in many respects determined or significantly influenced the way the thesis approaches the topic. Each institution accentuated different aspects of the work, which, naturally, affected the possibilities of the conducted research related to the thesis.

In order to present this experimental work in a more structured way, the thesis is predominantly organized in form of manuscripts. Thus, next to the chapters on **the history of corneal transplantation** (Chapter 1), **immunomodulatory approaches**

(Chapter 3) and **corneal transplantation models** (Chapter 4), the thesis contains the following chapters in a manuscript form:

- **Corneal immunology: a perspective on factors affecting alio- and xenografts (Chapter 2)**
  (K. Sedlakova, M. Filipec; submitted for publication)

- **The effect of the suturing technique on corneal xenograft survival (Chapter 5)**
  (K. Sedlakova, M. Filipec; submitted for publication)

- **FTY720 in corneal concordant xenotransplantation (Chapter 6)**
  (K. Sedlakova, E. Muckersie, M. Robertson, M. Filipec and J. V. Forrester)
  SEDLAKOVA K. et al. FTY720 in corneal concordant xenotransplantation. Transplantation, 2005, vol. 79, p. 297-303)

- **FTY720 in corneal allograft transplantation (Chapter 7)**
  (K. Sedlakova, M. Robertson, E. Muckersie, L. Duncan, M. Filipec, V. Holáň, and J. V. Forrester; in preparation)

- **FTY720 affects MHC class II expression on lymph node B cells (Chapter 8)**
  (K. Sedlakova, M. Robertson, E. Muckersie, L. Duncan, M. Filipec and J. V. Forrester; submitted for publication)

- **IL-4 and vIL-10 adenovirus-mediated *ex vivo* gene transfer in rat corneal allograft transplantation (Chapter 9)**
  (K. Sedlakova, H. Dannowski, M. Filipec, T. Ritter and U. Pleyer; in preparation).

Due to the manuscript form of the above-mentioned chapters, each pertinent chapter always respects the format required by the specific journal.

Thesis objective in short ...

- To study an experimental model of corneal transplantation in mouse and rat with the use of methods of cellular and molecular immunology.

- To establish an experimental corneal transplantation model in mouse and rat and grading schemas for the evaluation of the clinical course after grafting

- allotransplantation model (mouse to mouse; BALB/c to C57BL/6 and rat to rat; Wistar Furth to Lewis)

- concordant xenotransplantation model (rat to mouse; Lewis to BALB/c and Sprague Dawley to BALB/c).

- To assess the effect of the suturing technique on the survival of corneal concordant xenografts (Lewis to BALB/c) and the efficacy of selected immunosuppressants (CsA, monoclonal antibody against T cells (mAb anti-Thy-1.2) and AMT (selective inhibitor of iNOS)).

- To study the efficacy and the mode of action of FTY720 in the corneal concordant xenograft (Sprague Dawley to BALB/c) and allograft (BALB/c to C57BL/6) models and its mode of action;

- To evaluate the clinical efficacy of the IL-4 and vIL-10 adenovirus-mediated *ex vivo* gene transfer in rat corneal allograft transplantation (Wistar Furth to Lewis).

REFERENCES

1. WILLIAMS K. A., COSTER D. J. Penetrating corneal transplantation in the inbred rat: a new model. *Invest Ophthalmol Vis Sci*, 1985, vol. 26, p. 23-30.
2. SHE S. C., STEAHLY L. P., MOTICKA E. J. A method for performing full-thickness, orthotopic, penetrating keratoplasty in the mouse. *Ophthalmic Surg*, 1990, vol. 21, p. 781-785.
3. HASKOVA Z., FILIPEC M., HOLAN V. The role of major and minor histocompatibility antigens in orthotopic corneal transplantation in mice. *Folia Biol (Praha)*, 1996, vol. 42, p. 105-111.

Acknowledgments

During the work on my thesis, I cooperated with a number of people to whom I would like to express thanks.

First of all, my thanks go to Professor Martin Filipec and Associate Professor Vladimir Holáň, my supervisors, who provided me with the opportunity to conduct the studies in this interesting branch of science in the teams under their leadership and for a critical reading of the thesis.

I would also like to thank to Professor Uwe Pleyer and Dr. Thomas Ritter who introduced me to the field of gene transfer and under whose leadership I was given the possibility to perform a scale of various experiments one of which forms a part of the thesis.

My special thanks go to Professor John V. Forrester who enabled me to spend two wonderful years in a very stimulating and friendly environment at his Department of Ophthalmology in Aberdeen.

Further thanks must be expressed to all the technicians (in laboratories as well as in animal facilities) and administrative staff who were always very helpful and encouraging.

In addition, I would like to sincerely thank to my friends, Dr. Jan Schroeter, Dr. Vladimir Vladimirov and Ing. Petra Hřibová for their friendship and support.

Last but not least, I owe my deepest thanks to my family, especially my parents and my sister for their everlasting encouragement, help and support.

General introduction

Chapter 1

The past of corneal transplantation

Ophthalmology has never been separated from other medical specialisations and a number of advances in the field of corneal transplantation were a logical consequence of experiments performed earlier on other organ systems, while at the same time contributing to the overall knowledge of the transplantation rules itself.

The effort to preserve the sight accompanies the humanity from the remote past. Even today, despite the advances achieved during the last decades, the cornea and its diseases notably contribute to the prevalence of the blindness in the world, and millions of people suffer from cornea related blindness or severe sight restriction. Opacification of the cornea is unfavourable for several reasons. It alters the sight (depending on the location (central, paracentral, peripheral…) and partly on the density) and it impairs cosmetic appearance. Several surgical and non-surgical approaches were introduced.

A number of drugs were suggested to promote the absorption of corneal scars but none of them was generally accepted. A common characteristic of these remedies was their irritative nature. Traditionally, yellow oxide of mercury, noviform ointment, calomel powder, or jequirity were used and other remedies were described later (sodium chloride as subconjunctival injection (**August Rothmund** 1866 (*1*)), sodium salycilate (**Fromaget** and **Laffay**, 1897 (*2*)) or sodium and magnesium sulphate (**Thienpondt**, 1913 (*3*)). A further possibility was to use physical methods of treatment such as finger

massage (**Pagenstecher**, 1878-81 (*4, 5*)), galvanism (**Adler**, 1885 (*6*); **Hubert**, 1886 (*7*); **Alleman**, 1890 (*8*); **Pansier**, 1896 (*9*)), electric vibro-massage (**Maklkoff**, 1893 (*10*); **Piesbergen**, 1899 (*11*)), electrolysis (**Sulzer**, 1906 (*12*)) or x-rays and radium (**Sulzer**, 1906 (*12*); **Lawson** and **Davidson**, 1909 (*13*); **Flemming**, 1913 (*14*); **Koster**, 1913 (*15*)). The list might be much longer - other details can be found in chapter XIII, volume VII of Duke-Elder's System of Ophthalmology (*16*).

Notes about corneal surgery date from **Celsus** onward. **Galen** described complicated sutures, with or without superficial resection of the cornea, for the treatment of staphyloma and paracentesis and incision of the cornea for the treatment of hypopyon. In cases with central location of scar tissue, optical iridectomy was performed. However, the results were limited due to the irregularity of peripheral refraction. Alternatively, since the times of Galen scared corneas were tattooed in order to achieve at least some cosmetic improvement (he used copper sulphate reduced with nutgall). Tattooing not only helped cosmetically to improve the appearance but it could also reduce irregular light scattering caused by some nebulae by changing them into densely opaque plaques. In 1761, **Chevalier Taylor** described superficial keratectomy "to pare off the excrescence with a small curved knife, leaving as few irregularities as possible!".

Around 800 BC Indian physicians achieved mastery in reconstruction of a nose cut as punishment for thieves by translocation their skin from forehead. The following centuries brought scattered legends about transplantation attempts of breast, skin, or even leg. In Europe, the first notes about successfully performed transplantations appear in the 17th and 18th centuries. It is the 19th century, however, which marked the real expansion in experimental efforts performed both on animals and on humans. No

less important was the expansion of surgical knowledge on the anterior segment of the eye.

The methods used to fight the blindness due to opacification of the lens are handed over since ancient times. Advanced extraction of "thin" cataracts through the cornea or sclera performed by Arabian physicians (according to what Antyllos wrote) was substantially improved by **Ammar ibn `Ali al-Mawsili** who introduced a hollow needle through the sclera to suction an opaque lens. Unfortunately, in Western Europe this piece of knowledge was lost and had to be rediscovered in the 18th century. Before that, a sharp needle was used after a temporal paracentesis and opaque lens was displaced posteriorly (reclinated). Probably the first who intentionally started to extract cataracts through a corneal incision near the limbus was **Daviel** (1748). As is sometimes the case, it all initially began just as a rescue attempt of reclination surgery with accidental displacement of lens to anterior chamber, his own or of others (1722 **St. Yves** or **Petit**).

With the increasing experience in ophthalmic surgery, the possibility to replace the opaque cornea started to appear real. The first reference dates from the late 18th century. French physician **Guillaume Pellier de Quengsy** in his monograph *Précis ou Cours d´Opérations sur la Chirurgie des Yeux* in 1789 (the first monograph on ophthalmic surgery) (*17*) outlined the alternative replacement with convex glass slightly larger than the excised cornea, fitted in silver frame sutured by three sutures. The only evidence of a practical attempt is the note of **Guillaume-René Lefebure** about a woman from Bavaria to whom the artificial cornea was implanted in about 1792 but immediately failed. The surgeon was a brother of Professor Pellier (most likely **Denis**) (*18*). Guillaume mentioned one case performed by him in 1802 in his

unpublished manuscript *Avis au Peuple sur la Conservation de la Vue* with no further details about the outcome of the surgery. At about the same time, in 1795 **Erasmus Darwin** (grandfather of Charles Darwin) wrote, in a letter to his patient, about the possibility to explant a part of the cornea with an instrument similar to trephine with hope to get the cornea clear after healing. He also mentioned the idea to implant a piece of glass into the cornea in a shirtsleeve stud mode (*19*).

During the early 19th century, the majority of pioneering work in this field began in Germany - **Ferdinand von Graefe**, **Johann Friedrich Dieffenbach** in Berlin and **Philipp Franz von Walther** in Bonn and Munich (*20*). At the same time, sclerectomy for management of corneal opacities was attempted by others like **Johan von Autenrieth** (the opening was covered by a conjunctival flap that later opacified) (*21, 22*), **Johann Adam Schmidt** (*21*), **Georg Joseph Beer** (*22*), **Karl Himly** (*22*), **George James Guthrie** (*23*) or **Riecke** (*22*), **von Ammon** (*24*), **Weber** (*25*), **von Gärtner** (*22*) and **Pauli** (*26*). However, all these attempts were ultimately unsuccessful. There were also attempts to overcome the problem by bringing the clear part of the cornea to the line of vision (either by iridectomy or even by tenotomy to bring the eye in to new "central" position. Others tried excisions of corneal opacities with suturing.

In 1817, **Astley Cooper** performed a successful transplantation of the skin graft (**Baronio** applied skin autotransplantation on sheep in 1804) and **Franz Reisinger**, who attended this occasion in 1824, performed the first experimental corneal transplantation (in chicken and rabbits) and suggested replacing the opaque human cornea with a transparent animal cornea and named his technique "keratoplasty" (*27*). He did not use any trephine, just scissors and fixed the graft in place by direct sutures. He also noted that grafts healed in the recipient tissue although they became opaque

later. In addition, he performed subtotal and total penetrating auto and homografts in rabbits.

Although there exists some disagreement about who came first with the idea of the technique of corneal grafting, Franz Reisinger published his experiments first. One of the other possible candidates, his teacher **Karl Himly** asserted his priority "in no uncertain or polite terms" in 1813 (*28*) while, at the same time, also citing the recommendation proposed by **Johan von Autenrieth** in 1813 to dissect a window in the adjacent undiseased sclera and cover it with the clear cornea or conjunctiva. Another person claiming the priority was **Gottlieb Heinrich Mössner**.

In 1831, **Johannes Dieffenbach** failed in his attempts to graft the entire cornea when he sutured the donor tissue to the conjunctiva. Similarly, others like **Karl Himly** or **Philipp Joseph Drolshagen** who performed both alio- and xenografts (*29*) also failed - mainly because of imperfect instruments and inadequate technical skills or because of sepsis. In 1834, **Wilhelm Tohmé** in his thesis "*De Corneae Transplantatione*" described a new technique of corneal incision (still with a high rate of failures).

Samuel Bigger, who travelled to Germany – the then birthplace of corneal transplantation – in order to learn the new technique of keratoplasty, noted Tohmé`s report. In 1835, during his imprisonment by Bedouins in Egypt, he performed the first successful allotransplantation of the cornea in a pet gazelle and published a report on this operation two years later in Dublin (*30*). He suggested using pig corneas to replace the opaque tissue in humans and, one year later (1838) in New York, **Richard Sharp Kissam** transplanted a part of the cornea from a 6-month-old pig to James Dunn, a young Irishman with central leucoma. Although the graft remained in situ, two

weeks later it started becoming opaque and was absorbed within one month. He did not report on this enterprise until 1844 (*31*). In the same year, there was another xenograft experiment, now with the sheep cornea grafted into a human patient, performed by **Wutzer** (*32*). In both cases, the tissue healed-in but corneas became opaque. **Marcus**, in 1841, and **Steinberg**, in 1843, also experimented with animal-to-human cornea grafts - but failed (*33*). To sum up, between years 1837 to 1850, some of animal corneas transplanted to humans healed-in but all opacified and some eyes suffered panophthalmitis.

In the light of these unsuccessful results, using the lamellar graft became considered a more promising alternative. In 1840, **Philip Franz von Walther** (pupil of **Georg Joseph Beer**) came with the idea of triangular lamellar keratoplasty and, in the same year, **Mühlbauer** performed an experiment, on the basis of von Walther's ideas, with the sheep to human triangular lamellar grafts. He did not succeed either. In 1841, **Konigshofer** published a monograph in which he described the lamellar corneal graft as imagined by **von Walther** (*34*).

The limited success achieved with tissue grafts resulted in the attention being turned to the use of inert materials. In 1853, **von Nussbaum** (*35*) tried keratoprosthesis. He used gold, silver, and even copper, but eventually moved to glass (a 3 mm glass "collar" bottom shape button) which he transplanted to the rabbit cornea. However, all experiments were hampered by the infection or, three weeks later, the expulsion of the inserted material. Similarly unsuccessful results were reported by **Heusser** (graft retention for 3 months in a 19-year old woman) (*36*), **von Hippel** (*22*), **Dimmer** (*37*) or **Salzer** (9-33 months but in all cases with rethroprosthetic membrane formation and extrusion) (*38*).

In 1872, **Henry Power** reported his experience with corneal grafting on rabbits, dogs, cats, and even humans (he transplanted rabbit corneas into two children, but - as excepted – the grafted corneas became opaque and failed to function (*39*)). Because of the high rate of failure, Power started to realize that in order to achieve success he needed to keep attention to every detail. Thus, he considered not only the absence of infection or minimal trauma to the graft but also the use of freshly enucleated eye and the protection of the graft's endothelium as *condicio sine qua non*. Excellent was his observation that the adjacent tissue (sclera and conjunctiva) had to be left intact. He preferred, in contrast to **von Hippel**, the use of full-thickness keratoplasty with allografts from human eyes. Some of his grafts retained a certain degree of clarity.

The first human allotransplant was made by **Sellerbeck** in 1877, who used a graft from the eye of a 2.5-year old child with retinal glioma with an old but healed gonococcal infection. As a result, the patient could count fingers up to 3 metres several months after the surgery, the graft having a hazy central part (*40*).

J.R. Wolfe from Glasgow in 1879, similarly to **Power**, proved that only a graft of the same species would remain clear (*41*). In 1880, at the 48th Annual Meeting of the British Medical Association, Wolfe presented that it was not useful to attempt to transplant the whole cornea ("as soon as the cornea was removed, the lens and the vitreous humor came out, bleeding ensued, and loss of vision was inevitable, even if corneal transparency were secured"). He suggested that the use of the corneal oval from the centre, or little bellow the horizontal meridian, along with a conjunctival flap on each side assisting in keeping the graft in position was giving it a chance of

preserving its vitality. He presented the patient whose cornea was burnt by explosion 10 months after transplantation; the graft was vital and incorporated with the tissues, and "an amount of transparency and vision was beyond expectation") (*42*).

Joseph Lister´s concept of antisepsis in 1867, general anaesthesia by ether (1846) and chloroform (1847) and local anaesthesia by cocaine introduced by **Carl Koller** in 1884 enabled a considerable improvement of the operating conditions and results of corneal surgery. Another important contribution was the use of clockwork mechanical trephine invented by **Arthur von Hippel** in 1886, when he transplanted a full thickness rabbit graft as a lamellar graft in a girl and improved her vision from counting fingers to 6/60. In 1877, von Hippel grafted into a human a corneal disc of a dog - which clouded over (*33*). In 1886, he reported the first successful lamellar corneal graft (*43-46*). Some of his lamellar grafts remained clear and led to improved vision and he was the first to improve the vision permanently. Unfortunately, the use of animal donor materials prevented a possibly more favourable outcome. In his work he applied the principle of **Theodor Leber**, that transparency of the cornea is dependent on the integrity of endothelium in contact with the aqueous humor and Descemet membrane (1873) (*47*). **August Wagenmann** was the first to suggest that the failure of corneal transplantation is a result of the damage of endothelium (*48*). The first "lamellar" attempts (experimental and even a few clinical cases) were made by **Mühlbauer** in 1840 (*49*) followed by **Dürr** (1877) (*50*), but with no success. Eight years after **von Hippel**, in 1894, **Ernst Fuchs** already reported 30 cases of lamellar allotransplantation, and 2 full-thickness keratoplasties with some visual improvement. In contrast to von Hippel, he suggested that the opacification of the cornea after transplantation was caused by the cellular infiltration of the graft and not by the penetration of the aqueous humor to the graft (*51*).

Different sources for lamellas were used - material from the other patient's eye in 1908 by **Plange** (*52*), a clear part of the cornea from the periphery of the eye by **Morax** (1913) or the allograft by **Magitot** (1912) (*53*). In a period between 1910-1936, the technique was further improved and modified by others (**Löhlein** (*54*), **Elschnig** (*55, 56*), **Filatov** (*57*) and **Friede** (*58*)) but only the later work of **Paufique, Sourdille and Offret** established the technique of lamellar keratoplasty fully.

It is difficult to say who actually performed the first successful (i.e. which remained transparent) full-thickness corneal transplantation in human, but the acknowledgment for this goes to **Eduard Konrad Zirm**, an Austrian ophthalmologist working in Olomouc. On December 7th, 1905, he transplanted two grafts from a cornea of a 12-year-old boy who suffered a perforating scleral injury to a 45-year-old man with a corneal scarring due to lime burns. He used the trephine of **von Hippel**, but he modified the suturing technique to the overlay suture. He emphasized the importance of the usage of the tissue from a young human donor, the use of trephine, adequate anaesthesia with strict asepsis (but with no use of antiseptics), preoperative miosis by eserine (physostigmine), and the protection of the transplant between layers of gauze (*59*). After sixth months, the graft remained clear and the patient had visual acuity 3/50 for distance that enabled him to lead a normal life.

Another ophthalmologist who considerably contributed to the swing to the use of the now standard technique of full-thickness keratoplasty was the Prague school leader **Anton Elschnig**. Elschnig used various patterns of overlay sutures and, in 1930, suggested the use of full thickness inlay grafts. Importantly, he established in Prague the Center for Corneal Transplantations which was visited by a number of

ophthalmologists from the entire world (**Filatov** from Odesa, **Tudor Thomas, Ryckroft** and **Leigh** from Britain**, Löhlein** and **Friede** from Germany, **Franceschetti** and his collaborators from Switzerland and **Sourdille**, **Paufique and Ofrett** from France and **Castroviejo** and **Paton** from the USA). Among his students were **Ascher** (*60-62*), **Stanka** (*63*) or **Liebsch** (*64*). After the Second World War, his work continued in hands of **Jaromír Kurz**.

Although the viability of preserved corneas was confirmed already in 1911 by **Magitot** (*65*), in practice the use of preserved donor corneas was not accepted. It was not until 1935 when **Vladimir Petrovich Filatov** performed the first successful corneal transplant with a graft from a donor eye stored in a moist chamber up to 24 hours at 2 to 4 °C and popularized this technique (*57, 66*). He considered cadaver corneas up to 41 hours after death superior to a fresh tissue (*67*), while others preferred corneas as fresh as possible (i.e. up to 12-24 hours) - **Castroviejo** (1941), **Paufique** (1948) or **Franceschetti** (1949). Because of his contribution to the techniques of the donor eye preservation, he is considered a father of modern eye banking (*57, 66*). Inspired by the successful results of Elschnig´s school in Prague, **Filatov** contributed vastly to the popularization of perforating keratoplasty. Filatov recommends direct suturing, but when indirect sutures are used, he suggested using a reversed conjunctival flap or an egg membrane to keep the graft in place. Moreover, he also invented several surgical instruments.

The preservation of the donor cornea largely influences the outcome of keratoplasty. Donor corneas were preserved in various medias: for 8 days in haemolysed blood at 4°C (**Magitot**, 1912), in formalin that, not surprisingly, invariably became opaque (**Bürki**, 1948) or at 1-2°C in some suitable medium: blood plasma (**Magitot**, 1912),

saline (**Zirm**, 1906), olive oil (**Thomas**, 1931) or sterile liquid paraffin (**Bürki**, 1948). To reduce antigenicity, **Stocker** suggested storing the cornea in the recipient's serum in 1964. The material stored in this way could be used for up to 5-7 (or even 21 days) (**Buschke**, 1951). The initial attempts to use the cryopreserved corneas were unsuccessful, probably because of the destruction of endothelium by the freezing/thawing process (**Stocker**, 1953; **Leigh** and **Ridge**, 1957; **Leigh**, 1958). On the other hand, there is a report of **Eastcott** (1954) who successfully transplanted the cryopreserved human donor tissue. **Capella and Kaufman** developed the basic method of cryopreservation in 1965 (*68, 69*). In addition, vacuum dehydration in glycerine was demonstrated (**King**, 1957-58). Better results (equal to fresh material) were reached with frozen lamellar grafts – stored at -79°C in glycerol-saline mixture (**Eastcott,** 1954; **McPherson**, 1956). A very important step forward in the preservation of the cornea was the introduction of the MK medium in 1974 by **McCarey and Kaufman** (*70*) which enabled reliable storage of donor corneas for at least 3 to 4 days and thus made planning of surgery possible.

In the past, there were also attempts to transplant the entire cornea with a rim of conjunctiva and sclera but the results were not successful. The idea was suggested by **Wagenmann** in 1888 (*71*) and was tried by **Filatov** (*57*, *72*), **Burke** (*73*), **Elschnig** (*55*), **Key** (*74*), **Katz** (*75*) and **Rycroft** (*76*). In all the cases, grafts became opaque and the eye degenerated through phthisis or secondary glaucoma. Better results were attained only when the entire cornea was transplanted by **Castroviejo** (1950-51) (*77, 78*), although the operation required a complete removal of the iris with the lens. There were also unsuccessful attempts to transplant the entire anterior segment of the eye by **Schimanovski** in 1912 (*79*) and later by **Filatov** in 1935 (*57*) or **Maumenee** (*80*).

Gradual acceptance of perforating corneal transplantation was associated with the effort to improve surgical techniques and to achieve a higher rate of success. New models of trephines or donor cornea punches, different size or shape of the grafts, suturing techniques – all that was discussed at that time by, for instance, **Löhlein**, **Filatov**, **Nizetic**, **Franceschetti**, **Vannas**, **Ramon Castroviejo**, **Weiner**, **Tai** or **Aruga**. By the end of 1930s, keratoplasty was accepted as relatively successful in carefully selected cases also thanks to the introduction of antibiotics and corticosteroids in 1940s, small fine Swiss needles with 8-0 sutures and an operating microscope (**Richard Troutman**). The introduction of the fine suture silk material also allowed departure from the use of overly sutures.

To cope with the increasing demand for donor corneas for grafting, **Townly Paton** set up in New York in 1959 the first eye bank - **The Eye Bank for Sight Restoration**. Later, in 1961, the intention to "lay down the standards for obtaining, storage, preservation, and usage of donor tissue among the different eye banks" led to the establishment of the **Eye Bank Association of America.** The invention of the specular microscope in 1968 by **David Maurice** provided the means of studying donor and transplanted corneal endothelium and enabled the elimination of unsuitable donor material.

In recent years the interest in the concept of lamellar corneal transplantation has become intensive again, now not only for the restoration of the superficial corneal layers, but also of the endothelial side in patients with corneal endothelial dysfunction. The new technique of the "posterior lamellar keratoplasty" (PLK) has already several modifications but the principle remains the same: the posterior lamellar graft is inserted through a scleral incision and left unsutured. **Gerrit R. J. Melles**, one of the

pioneers in the field of PLK summarized the advantage of this approach: "*Compared with a penetrating keratoplasty, all these techniques may give minimal change in refractive power of the transplanted cornea, minimal induced astigmatism, elimination of suture-induced complications, elimination of late wound dehiscence, faster visual rehabilitation, and a lesser demand of postoperative aftercare.*" (*81*). Although we are still in the process of seeking the optimal surgical technique, there are already some optimistic short term results (*82, 83*) and, importantly, the loss of corneal endothelium seems to match the loss after full thickness corneal transplantation after the 3 years follow-up (*83*).

Last fifty years represented the period of other technical improvements (better operating microscopes, finer suture material (monofilament nylon), finer needles, microsurgical instruments, or the introduction of sodium hyaluronate derivates to protect the delicate endothelium). However, most importantly, it was also the time of significant extension of our knowledge of corneal physiology and of transplantation immunology. In addition, the time marked great progress in immunosuppressive therapy. Nevertheless, we still need to cope with adverse effects of such therapy or with its ineffectiveness in a number of cases. If all approaches fail, the use of keratoprosthesis remains the ultimate resort.

REFERENCES

1. ROTHMUND. *Klin Monatsbl Augenheilkd*, 1866, vol. 4, p. 161.
2. FROMAGET, LAFFAY. *Ann. Oculist. (Paris)*, 1897, vol. 118, p. 43.
3. THIENPONDT. *Clin. Ophthal.*, 1913, vol. 5, p. 569.
4. PAGENSTECHER. *Zbl. Prakt. Augenheilk.*, 1878, vol. 2, p. 281.
5. PAGENSTECHER. *Arch. Augenheilk.*, 1881, vol. 10, p. 225.
6. ADLER. *Wien. Med. Wschr.*, 1885, vol. 35, p. 560.
7. HUBERT. *Bull. Soc. Franc. Ophthal.*, 1886, vol. 4, p. 118.
8. ALLEMAN. *Brooklin Med. J.*, 1890, vol. 4, p. 769.
9. PANSIER. Traité d´éléctro-thérapie oculaire. 1896.
10. MAKLKOFF. *Arch. Ophtal. (Paris)*, 1893, vol. 13, p. 530.
11. PIESBERGEN. *Zbl. Prakt. Augenheilk.*, 1899, vol. 23, p. 33.
12. SULZER. *Ann. Oculist. (Paris)*, 1906, vol. 136, p. 349.
13. LAWSON, DAVIDSON. *Trans. Ophthal. Soc. U.K.*, 1909, vol. 29, p. 306.
14. FLEMMING. *Graefes Arch. Ophthal.*, 1913, vol. 84, p. 345.
15. KOSTER. *XII Int. Cong. Med., London*, 1913, vol. 9, p. 241.
16. DUKE-ELDER S. The foundations of ophthalmology. Heredity, pathology, diagnosis and therapeutics., 1996, vol. 7, p. Chapter 23.
17. DE QUENGSY G. P. Précis au cours dóperations sur la chirurgie des yeux. *Didot (Paris)*, 1789, vol. 1, p. 94.
18. REMEKY H. Les fréres Pellier et la kératoprosthése. Mémoires Bull. de la Soc. Francophone d´Histoire de l´Ophthalmologie. 1996-1997, vol. 4, p. 53.
19. KING-HELE D. The letters of Erasmus Garwin. *Cambridge University Press*, 1981, p. 276.
20. SEBASTIAN G. [150 years of the "Handbook of plastic surgery"--in memory of Eduard Zeis (1807-1868)]. *Hautarzt*, 1989, vol. 40, p. 45.

21. SCHMIDT I. De pupilla artificialis in sclerotica aperienda. *Tübingen*, 1814.

22. VON HIPPEL A. Uber die operative Behandlung totaler stationärer Hornhaut Trübungen. *Arch F Ophthalmol*, 1877, p. 79.

23. GUTHRIE G. A treatise on the operation for the formation of an artificial pupil. 1819.

24. VON AMMON A. Die Sclerectomie oder die künstliche Pupillenbildung in der Sklerotica. *Z Augenheilkd*, 1831, vol., p. 183.

25. WEBER L. Dissert. Inaug. sistens observationes quasdam in coretodialysiu et pupillam in sclerotica aperiendam. *Tübingen*, 1817.

26. PAULI. Uber Keratoplastik und Sclerectomie. *Schmidt CC, ed. Jahrbücher der in- und auslandischen gesammten Medizin; Leipzig : Otto Weigand*, 1883.

27. REISINGER F. R. Die Keratoplastik, ein Versuch zur Enweiterung der Augenheilkunde. *Bayerische Ann. Chir. Augenheilk.*, 1824, vol. 1, p. 207.

28. HIMLY K. Die Krankenheiten u. Missbildungen d. mensch. Auges (Berlin). 1843, vol. 2, p. 44.

29. DROLSHAGEN P. De vulnerabilitate oculi. *Berlin*, 1834.

30. BIGGER S. L. L. An inquiry into the possibility of transplanting the cornea with a view of relieving blindness. *Dubl J Med Sci*, 1837, vol. 11, p. 408.

31. KISSAM R. S. Ceratoplastice in man. *New York J. Med.*, 1844, vol. 2, p. 281.

32. RYCROFT B. The scope of corneal grafting. *Br J Ophthalmol*, 1954, vol. 38, p. 1.

33. GORIN G. History of ophthalmology. Publish or perish., 1982.

34. KÖNIGSHOFER T. De transplantatione corneae (Opus praemio ornatum, Monachii, 1841). *Jarbucher der in- und auslandischen gesammten Medicin*, 1843, vol. 27, p. 128.

35. NUSSBAUM V. Cornea artificialis. *Deutsche Klinik Berlin*, 1853, vol. 5, p. 367.

36. HEUSSER. *Oesterreich. Z. prakt. Heilk.*, 1860, vol., p. 424.

37. DIMMER F. Zur operativen Behandlung totaler Hornhautnarben mit vorderer Synechie: Bericht uber die zwanzigste Versammlung der Ophthalmologischen Gesellschaft. *Heidelberg: Ferd. Enke Verlag.*, 1889, p. 147.

38. SALZER F. Uber kunstlichen Hornhautersatz. *Ber. Deutsche Ophth Gesellschaft*, 1895, vol. 24, p. 230.

39. POWER H. *Proc. of IV. Int. cong. ophthal., Saville, Edwards & Co., London*, 1872, vol. 4, p. 172.

40. SNYDER C. Alois Glogar, Karl Bräuer, and Eduard Konrad Zirm. *Our Ophthalmic Heritage*, 1967, p. 107.

41. WOLFE J. R. *Med Times Gaz*, 1879, vol. 2, p. 579.

42. WOLFE J. R. *The British Medical Journal*, 1880, vol. Nov. 13, p. 780.

43. VON HIPPEL A. Über die operative Behandlung totalen stationar Hornhauttrubungen. *Graefes Arch. Ophthal.*, 1877, vol. 23, p. 79.

44. VON HIPPEL A. *Graefes Arch. Ophthal.*, 1878, vol. 24, p. 235.

45. VON HIPPEL A. On transplantation of the cornea. *Ophthalmol. Gesellschaft Heidelberg 18 (2nd Meeting)*, 1886, p. 54.

46. VON HIPPEL A. *Graefes Arch. Ophthal.*, 1888, vol. 34, p. 108.

47. LEBER T. *Graefes Arch. Ophthal.*, 1873, vol. 19, p. 87.

48. WAGENMANN A. Experimentelle Untersuchung zur Frage der Keratoplastik. *Arch F Ophthalmol*, 1888, p. 211.

49. MÜHLBAUER. Über d. Transplantation d. Hornhaut (Munich). 1840.

50. DURR. *Klin Monatsbl Augenheilkd*, 1877, vol. 15, p. 305.

51. FUCHS E. Zur Keratoplastik. *Z Augenheilkd*, 1901, vol. 5, p. 15.

52. PLANGE. *Klin Monatsbl Augenheilkd*, 1908, vol. 46, p. 277.

53. MAGITOT. *Ann. Oculist. (Paris)*, 1912, vol. 147, p. 44.

54. LOHLEIN W. *Arch. Augenheilk.*, 1910, vol. 67, p. 398.

55. ELSCHNIG A. Augenärzt. Operationslehre. *Graefe-Saemisch Hb. d. ges. Augenheilk. 3rd ed.*, 1922, vol. 1, p. 514.

56. ELSCHNIG A. *Arch. Ophthalmol. (Chicago)*, 1930, vol. 4, p. 165.

57. FILATOV V. P. Transplantation of the cornea. *Arch. Ophthalmol. (Chicago)*, 1935, vol. 13, p. 321.

58. FRIEDE. *Arch. Augenheilk.*, 1936, vol. 109, p. 243.

59. ZIRM E. K. Eine erfolgreiche totale Keratoplastik. *Arch F Ophthalmol*, 1906, vol. 64, p. 580.

60. ASCHER. *Graefes Arch. Ophthal.*, 1919, vol. 99, p. 339.

61. ASCHER. *Graefes Arch. Ophthal.*, 1922, vol. 107, p. 439.

62. ASCHER. *Graefes Arch. Ophthal.*, 1923, vol. 111, p. 446.

63. STANKA. *Graefes Arch. Ophthal.*, 1927, p. 335.

64. LIEBSCH. *Arch. Augenheilk.*, 1930, vol. 103, p. 603.

65. MAGITOT A. Recherches expérimentales sur la survie possible de la cornée conservée en dehors de l´organisme et sur la kératoplastie différée. *Ann. Oculist. (Paris)*, 1911, vol. 146, p. 1.

66. FILATOV V. P., BAJENOVA M. A. Culture of dried corneal tissue. *Rev Gen Ophth*, 1937, vol. 1, p. 385.

67. FILATOV V. P. La corneé de cadavre comme material de transplantation. *Ann. Oculist. (Paris)*, 1934, vol. 171, p. 721.

68. CAPELLA J. A., KAUFMAN H. E., ROBBINS J. E. Preservation of viable corneal tissue. *Cryobiology*, 1965, vol. 2, p. 116.

69. MCCAREY B. E., KAUFMAN H. E. Improved corneal storage. *Invest Ophthalmol*, 1974, vol. 13, p. 165.

70. LINDSTROM R. L. Advances in corneal preservation. *Trans Am Ophthalmol Soc*, 1990, vol. 88, p. 555.

71. WAGENMANN A. *Graefes Arch. Ophthal.*, 1888, vol. 34, p. 211.

72. FILATOV V. P. *Vestn Oftal*, 1913, vol. 30, p. 534.

73. BURKE. *Arch. Ophthalmol. (Chicago)*, 1921, vol. 50, p. 147.

74. KEY. *Trans. Amer. Ophthal. Soc.*, 1930, vol. 28, p. 29.

75. KATZ. *Arch. Ophthalmol. (Chicago)*, 1933, vol. 9, p. 331.

76. RYCROFT B. W. *Br J Ophthalmol*, 1954, vol. 38, p. 1.

77. CASTROVIEJO R. *Ann. Oculist. (Paris)*, 1950, vol. 183, p. 641.

78. CASTROVIEJO R. *Am J Ophthalmol*, 1951, vol. 34, p. 1697.

79. SCHIMANOVSKI. *Vestn Oftal*, 1912, vol. 29, p. 812.

80. MAUMENEE A. E. Penetrating autokeratoplasty of entire cornea. *Am J Ophthalmol*, 1959, vol. 47, p. 125.

81. MELLES G. R. Posterior lamellar keratoplasty: DLEK to DSEK to DMEK. *Cornea*, 2006, vol. 25, p. 879.

82. SANO Y. Corneal endothelial transplantation: results of a clinical series using deep lamellar endothelial keratoplasty (DLEK). *Cornea*, 2004, vol. 23, p. S55.

83. VAN DOOREN B. et al. Endothelial cell density after posterior lamellar keratoplasty (Melles techniques): 3 years follow-up. *Am J Ophthalmol*, 2004, vol. 138, p. 211.

Chapter 2

Corneal immunology: a perspective on factors affecting alio- and xenografts

Klara Sedlakova, Martin Filipec

Perspective (Review)

Submitted for publication

Perspective (Review)

Corneal immunology: a perspective on factors affecting alio- and xenografts

Klara Sedlakova MD[1], Martin Filipec PhD[2, 3]

[1] Department of Ophthalmology, 1st Medical Faculty of Charles University and General Faculty Hospital, Prague, Czech Republic

[2] Laboratory of Ophthalmology and Eye Bank, 1st Medical Faculty and General Faculty Hospital, Charles University, Prague, Czech Republic

[3] Eye Clinic Lexum, Prague, Czech Republic

Address correspondence to: M. Filipec, Eye Clinic Lexum, U Společenské zahrady 3, Prague 4 - Krč, Czech Republic. Phone: (+420) 244 016 441, Fax: (+420) 244 016 440; email: murfi@centrum.cz.

Running title: Immunology of corneal transplantation

ABSTRACT

For a long time, the cornea was considered sequestrated from (and ignored by) the immune system. Specific features collectively addressed as "immune privilege" help protect the cornea from the immune system through manipulating regional innate and adaptive immunity. However, it is known that the immune privilege is only relative and despite many inhibitory mechanisms, corneal grafts are still rejected. In this review, we analyze current evidence based on recent advances in basic and clinical research. In addition, we provide an analysis of factors which affect corneal allo- and xenografts.

Key words: corneal transplantation, allotransplantation, xenotransplantation, immune privilege, rejection

INTRODUCTION

The outcome of human corneal transplantation after one year is generally excellent (about 90% of graft survival),[1,2] despite the fact that non human leukocyte antigens (HLA)-matched grafts are predominantly used. However, the probability of graft survival (normal- and high-risk) is 0.73 at 5 years, 0.62 at 10 years, and 0.55 at 15 years, which may be less than the equivalent rate for some vascularized grafts. This is especially true in cases marked by corneal neovascularization, active inflammation, or a previous history of corneal graft rejection, all considered to be “high-risk” grafts. According to a report by Williams *et al.*[1] based on records of 10,952 full-thickness corneal grafts, there are several factors predictive of the corneal graft failure: "transplant centre, donor age, preoperative diagnosis, number of previous ipsilateral grafts, lens status, history of corneal neovascularization, ocular inflammation or raised intraocular pressure in the grafted eye, requirement for anterior vitrectomy, graft size, early suture removal, postoperative events including graft neovascularization, rise in intraocular pressure, rejection episodes, type of treatment for raised intraocular pressure, arrangements for recipient follow-up and further eleven factors". Notably, there has been no improvement in graft survival over a 15-year time period.

THE IMMUNE PRIVILEGE OF THE CORNEA

For a long time, the cornea together with the brain have been considered to be sequestered from (and ignored by) the immune system because of the absence of lymphatic drainage and because of their existence behind the blood–ocular or –brain barrier. The cornea enjoys the advantage of an immune privilege and there are a number of factors which help to protect it from the immune system.[3] The major factors contributing to immune privilege are:

- Corneal avascularity – under normal conditions the cornea is devoid of blood and lymphatic vessels with the exception of the limbal area. Blood and lymphatic vessels can be induced *de novo* by inflammatory stimuli. It takes lymphatic vessels about six months to regress and the period is even longer for blood vessels in mouse.[4]

- Blood ocular barrier - unless compromised by the pathological/inflammatory process, it prevents migration of immune cells from blood to the anterior chamber.

- The relative absence of lymphatic drainage (antigens injected intravenously/intracamerally may be less immunogenic than those introduced by other routes).

- Constitutive expression of Fas ligand (FasL) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) by corneal endothelial cells (induces apoptosis of Fas-bearing cytotoxic T cells[5] and some other cell populations.[6,7]

- Constitutive expression of B7-H1 in the cornea, iris and ciliary body (also in retina) - induces apoptosis of the programmed death - 1 (PD-1) bearing T cells.[8]

- Low levels of the expression of major histocompatibility complex (MHC) class I molecules on the surface of corneal endothelial cells helps protect cells from T cell-mediated tissue injury but can leave them vulnerable to natural killer (NK)-mediated lysis.[9,10] On corneal endothelium next to classical MHC class I was

detected the expression of non-classical MHC class Ib molecules Qa-2.[11] Experimental data using melanoma cells suggest that Qa-2 may serve as a ligand for yet unidentified NK inhibitory receptor(s) expressed on NK1.1(+) NK/T cells[12] and balance the problem raised by MHC class I deficiency. In contrast to MHC class I, MHC class II molecules are induced de novo and are strongly expressed on endothelium during rejection in contrast to MHC class I.[10]

- Anterior chamber-associated immune deviation (ACAID), a form of peripheral immune tolerance was described already in 1873 by Van Dooremal and further elaborated by Medawar. Later Kaplan and Streilein inoculated mice intracamerally with P815 tumor cells and observed that these cells not only grow progressively but also elicit a systemic antibody response that was in sharp contrast to the accepted immune ignorance theory. At the same time, this approach prolonged survival of DBA/2 skin allografts suggesting its systemic effect. Importantly, this mechanism was dependent on the presence of not only the spleen, but also the thymus, B cells, B cell receptor, CD25+, IL-10-producing CD4+T cells or $\gamma\delta$ T cells, transforming growth factor-beta (TGF-β) (respectively IL-10)[13] or, for example, intact sympathetic nervous system. Antigens when injected into the anterior chamber (AC) induce (via F4/80+ antigen presenting cells (APC) or in soluble form?) within 24 hours activation of natural killer (NK) 1.1+ CD4-CD8-T cell receptor $\alpha\beta$+ immunoregulatory thymocytes. These cells are during two to five days exported to the peripheral lymphoid tissues[14-16] where they activate CD8+T-splenic suppressor cells.[16] Antigens also reach the spleen (via F4/80+APC or in soluble form?) where they elicit the generation of Ag-specific B cells that induce both CD4+ and CD8+ regulatory T cells (Tregs).[17] The nature of the antigen seems to play some role,

as well, namely whether the antigen injected in the eye is soluble or in a particulate form. Particulate antigens preferentially induce IL-12 production by responding APC in contrast to IL-10 induced by soluble antigens.[13] These tolerogenic APC express high levels of macrophage inflammatory protein-2 that recruit natural killer T (NKT) cells to the splenic marginal zone via CXCR2.[14] The exact origin of F4/80+APC is unknown. Some authors support the hypothesis that cells are of iris, ciliary body[18] or trabecular meshwork origin.[19] However, one can speculate about their extraocular location. Experiments by Wang *et al.* show that after antigen inoculation into AC, even blood mononuclear F4/80+CD1+ cells can induce regulatory thymocytes and ACAID.[14] This may indicate the importance of the direct route of antigen to venous circulation and consequently to the thymus and the spleen. The ACAID inducing properties are not solely attributed to AC (or more precisely to properties of the aqueous humor or cultured iris and ciliary body cells supernatants) but can be induced by amniotic or cerebrospinal fluid (fluids of other immune privileged sites that all contain latent and/or active TGF-β).[20] Importantly, even intravenous injections of soluble antigen may induce immune deviation[21] and as little as twenty F4/80+ cells have been able to induce ACAID.[22]

ACAID has been for a long time understood as affecting solely delayed-type hypersensitivity (DTH) and not humoral immunity. This selective impairment has recently been questioned, for example, by experiments of Wilbanks *et al.* in which inoculation of soluble antigen into AC or intravenously leads to insufficient production of complement-fixing (IgG2) antibodies by bovine serum albumin-specific B cells.[21]

- Immunosuppressive properties of the aqueous humor (reviewed by Taylor[23])

  - TGF-β - inhibits T cell activation and differentiation (APC when pretreated with TGF-β and later pulsed with antigen induce antigen-specific suppression of DTH and when adoptively transferred intravenously, induce antigen-specific immune deviation of ACAID type[13]). In addition, for example TGF-β inhibits activation of neutrophils by Fas ligand (FasL, CD95 ligand) or NK cells.

  - Neuropeptides (alpha-melanocyte-stimulating hormone, vasoactive intestinal peptide, calcitonin gene-related peptide, migration inhibitory factor) - neural control of immune privilege - inhibit and alter the functional properties of T lymphocytes, NK cells and macrophages.[24,25]

  - Aqueous humor contains complement inhibitory factors (probably the soluble forms of membrane cofactor protein, decay-accelerating factor, and CD59).[26]

The importance of the immune privilege seems to be predominantly in avoiding (or rather tapering) the inflammatory response induced by trauma or invading pathogens and in limiting its destructive effect on the visual axis where only very low threshold for anatomical distortion exists. However, the immune privilege is only relative and despite so many inhibitory mechanisms, corneal grafts are still rejected.

THE IMMUNE RESPONSE - AFFERENT ARM

After corneal transplantation, the induction of the immune response includes presentation of corneal antigen to naïve T and B cells, which results in their priming, activation and clonal expansion in draining lymph nodes (DLN) and the spleen. Antigens are transported to secondary lymphoid organs (in addition to the thymus) in a soluble form or by professional antigen presenting cells.

Corneal antigens

The role of major histocompatibility antigens

The antigens presented in the context of MHC are responsible for tissue incompatibility and for prevention of donor tissue acceptance between non-related individuals of the same species. While the expression of MHC class I antigens was detected on corneal epithelial, stromal, and endothelial cells,[27] the expression of MHC class II antigens is relatively low, localized either on Langerhans cells in the epithelium[28] or present on dendritic cells throughout the corneal stroma (mainly near the limbus). Stimulation by interferon-gamma (IFN-γ) induced MHC class II expression on endothelial and stromal corneal cells[29] while in other reports the combination of IFN-γ and tumor necrosis factor-alpha (TNF-α) was necessary for their induction on corneal endothelial cells.[30]

The importance of MHC matching and its effect on graft survival are well documented in the case of other organs. In corneal transplantation, however, this issue is controversial and still awaits resolution. Previous experiments gave rise to opposite results in some instances, although most of them did show some benefits. Because of such contradictory results, the conclusion was that in corneal

transplantation HLA matching is not important. However, recent experimental data imply that both HLA class I and II matching play an important role in normal- and also high-risk situations. The use of HLA matched corneas (HLA-A, -B or –DR) reduced the rejection rate by at least 10% 10 years after transplantation in normal-risk and by 40% in high-risk patients after 3 years.[31] According to Khaireddin *et al.*[31] especially HLA-B mismatches represent important prognostic factors for both normal- and high-risk patients.

A possible explanation of contradictory results these studies produce is summarized by Reinhard *et al:*[32] and similarly by Wachtlin *et al.:*[33] a) In large multicentre studies, different levels of surgical experience or technique may affect the outcome of corneal transplantation. b) HLA matching does not influence additional risk factors such as surface disorders, glaucoma in high-risk recipients who were mainly involved. c) Most older studies performed HLA typing serologically and not by DNA typing and this fact could result in errors (for example, in the Collaborative Corneal Transplantation Studies the occurrence of typing errors was up to 25%).[34]

The role of ABO and Lewis antigens

The A, B, H(O) and Lewis antigens were originally detected on erythrocytes but their expression is much wider.[35] It is a family of molecules that differ biochemically and genetically. Their expression was detected on corneal epithelium[36] and conjunctiva,[37] but the expression on corneal endothelium remains controversial (present[36,38] x absent[27,39]). In contrast to solid organs, in corneal transplantation ABO-incompatibility seemed to play a rather minor role,[40,41] but, there are studies with different results.[2,42] The explanation may be provided by *in vitro* experiments of Chan *et al.*[43]

demonstrating that for the lysis of corneal epithelial cells, a relatively high level of antibodies is needed (a level similar to that found in serum). Since their level in tears or aqueous is relatively low, unless blood ocular barrier is broken, ABO-incompatibility does not influence the outcome significantly. The role of Lewis antigens in corneal transplantation was shown to be important in normal-risk recipients (84% vs. 68% rejection rate for Lewis compatible patients).[41] In conclusion, the importance of ABO and Lewis antigens matching has not been resolved yet and requires further research.

The role of minor histocompatibility antigens (mHA)

The existence of additional histocompatibility loci was revealed by experiments with inbred mice that rejected skin grafts or tumor cells from H2-identical, "background"-disparate strains. Similarly, in humans, rejection of skin grafts was observed between HLA-identical siblings. Later on, minor histocompatibility antigens were identified as specific peptides recognized by donor T cells after HLA-matched allogeneic hematopoietic stem cell transplantation.

MHA are encoded by genes outside the MHC and include certain intracellular proteins encoded by mitochondrial DNA, Y or autosomal chromosomes. They can be presented by both MHC class I and class II molecules and are recognized by CD4+T cells, CD8+ as well as by B cells. Although in many cases their chromosomal locations have been determined, the antigens themselves remain mostly uncharacterized. MHA are supposed to be the primary antigenic targets of the graft versus host disease (GVHD). Ten-year survival of kidney grafts is only 40% despite MHC-matching and there is 20–30% risk of rejection between HLA genotypically identical siblings. This is believed to result from mHA mismatches.[44] Many mHA are tissue-restricted and, thus, only specific tissues may be at risk for rejection. Interestingly, the clinical picture of

mHA GVHD after bone marrow transplantation resembles several autoimmune diseases (systemic lupus erythematosus and scleroderma).[45]

It remains to identify how many and which mHA will be immunodominant in a transplantation setting. The transplantation barrier between C57BL/6 and BALB/B mice may consist of at least 40 minor histocompatibility antigens.[46] Very little is known about their role in corneal allograft transplantation. There is a suggestion from rodent model that they may be an important barrier against acceptance[47-49] (for example donor corneas expressing H3b, which are recognized by CD4+T cells, were rejected even faster than skin allografts)[48] which may reflect reduced level of MHC expression on corneal tissue.[48] Recent report based on rodent model suggests that matching for the minor histocompatibility antigen HLA-A1/H-Y may improve prognosis in human corneal transplantation.[50]

Antigen presenting cells

APC of corneal epithelium

In mouse corneal epithelium, there resides an important population of professional antigen presenting cells - Langerhans cells (LC), an epithelial form of DC (in the corneal periphery MHC class II+, and MHC class-population in the central area of the normal cornea). Under inflammatory conditions MHC class II and CD80/CD86 costimulatory molecules are upregulated.[51] Their presence in the central cornea has implications for considering a direct pathway of naïve T cell sensitization in addition to an indirect pathway of allorecognition that predominates.

The potential of LC to influence corneal graft survival seems to be very important. Experiments of Hori *et al.* in both normal- and high-risk (prevascularized) mouse corneal allotransplantation show that a composite graft containing host epithelium has a survival rate of more than 85% vs. 40–50% in controls.[52,53] The survival rate decreases when previously cauterized syngeneic epithelium is used. This may suggest, firstly, the important role of epithelium as a strong immunogen and, secondly, the importance of total amount of antigens with respect to their immunogenicity presented in DLN. This notion gains support from a clinical study where loss of corneal LC, during storage in organ culture medium may contribute to initially high acceptance of corneal grafts in humans as suggested by results of Ardjomand *et al.* (91 vs. 73% in normal-risk and 70 vs. 35% in high-risk recipients after 18 months).[54,55]

APC of the corneal stroma

The central part of the corneal stroma has been for a long time considered to be devoid of MHC class II positive dendritic cells unless being under inflammatory conditions. This has been recently questioned by Hamrah *et al.*[56] who demonstrate six resident bone marrow derived subsets of dendritic cells (DC) in mouse. In the central cornea, they detected DC and LC, which do not express MHC class II molecules and co-stimulatory molecules, and in the anterior periphery, mature MHC class II, costimulatory molecule (CD80+ and CD86+) expressing CD11c+DC and LC. Macrophage-like cells were detected in the posterior part and CD14+ cells throughout the whole cornea. However, these results contrast with those of other researchers who failed to detect significant numbers of DC in the central cornea;[57,58] but detected different subpopulations of CD45+CD11b+ cells. They comprised about 3-4% of total stromal cells; half of them were F4/80+ (30% of the total number and 50% of F4/80+ cells were also MHC class II+).[57]

The situation differs under inflammatory conditions under which a significant increase in numbers of DC (with upregulated expression of costimulatory and MHC class II molecules) and CD11c-CD11b+ cells of monocyte/macrophage origin occurs.[59]

APC in the anterior uveal tract

The anterior uveal tract also contains populations of cell with phagocytic and antigen presenting capabilities. There are iris and ciliary body dendritic cells (CD11c+ mainly F4/80+ MHC class II+ weakly CD80 and CD86+) and macrophages (CD11b+F4/80+CD11c-).[60-62] The majority of antigens from the AC are directed to the trabecular meshwork where APC and phagocytic cells also reside. Experiments of Dullforce *et al.* suggest that APC from the anterior uveal tract do not migrate to DLN or to the spleen[19] and that the majority of antigens that reach these organs via vascular and lymphatic routes are in a soluble rather than cell-associated form.[63,64] The question of whether APC from trabecular meshwork are involved must be analyzed further.

Antigen trafficking to secondary lymphoid tissues (DLN and the spleen) and the thymus

Following an intracameral injection, antigens get access (via trabecular meshwork) mainly to blood and subsequently the spleen primarily in a soluble form[65]. Presumably, the same applies in the case of the thymus. In addition to this "conventional" aqueous outflow pathway, there is another "unconventional" one, which enables antigens to reach lymph nodes of the head and neck and, via venous circulation, mesenteric

lymph nodes.[64,66] It is estimated that this uveoscleral pathway in mouse represents about 16% of the total outflow.[67]

After subconjunctival injection, up to 97% of antigens are drained via lymphatics to DLN – to submandibular and in smaller extent to cervical superficial lymph nodes (predominantly ipsilaterally).[67] The exact route of antigen/APC from the cornea to the DLN has not been fully explained yet. It is possible that corneal APC can access conjunctival lymphatics by migration through the limbal area. Under inflammatory conditions, which stimulate vasculo- and lymphangiogenesis, the situation is more obvious. The injection of ^{131}I-albumin-Evans blue complex into the center of the lymph/vascularized rat cornea leads to the presence of the dye in DLN in six minutes in contrast to about six hours (and one thousandth fraction) by diffusion in the normal cornea.[68] Similarly, donor derived corneal CD11c+, MHC class II+DC were detected in DLN but their origin has not been described.[66]

In dermis, after soluble antigen injection, the first wave of antigens arrived to the DLN within 30 minutes in a free form (capable of initiating T cell proliferation, but not DTH to additional challenge). The second wave arrived in between 18–24 hours, together with dendritic cells which allowed sustained expression of the IL-2 receptor and supported DTH.[69] However, similar data are not yet available for the cornea.

Antigen presentation - direct and indirect allorecognition

In solid organ transplantations, direct allorecognition, which characterizes direct recognition of foreign MHC complexes on donor cells (in particular on donor APC) by recipient T cells, has a major role in the early post-transplant period. Later on, when donor APC from the graft are depleted (usually within weeks after transplantation), the

indirect type takes over and may supply antigens responsible for chronic rejection. It is based on CD4+T cell recognition of processed donor peptides as well as MHC class molecules on the surface of self-APC.

The situation in corneal transplantation seems to be different. The central part of the cornea (which corresponds to the area of the graft) was for a long time considered to be devoid of APC and thus unable to mount direct presentation of antigens in DLN. Accordingly, the indirect mode of allorecognition mediated by CD4+T cells was accepted as dominant in contrast to other organ transplantations in normal-risk recipients. There are several experimental data supporting it.[70]

The situation differs under high-risk conditions (i.e. when the graft is placed in an inflamed vascularized corneal bed) where donor MHC class II deficiency delays graft rejection. CD4+T cell-mediated direct allorecognition thus contributes to the indirect pathway in contrast to uninflamed corneal beds.[71] Similarly, composite grafts containing syngeneic epithelium which were two weeks before enhanced for the presence of LC by light cauterization are swiftly rejected in contrast to allogeneic composite grafts with syngeneic epithelium alone.[53] It shows, firstly, the important role of LC in direct or indirect allorecognition and graft rejection, which is proportional to their number and, secondly, the significance of corneal epithelium in promoting graft rejection. Under normal conditions with unaltered LC numbers, the amount of alloantigen from composite grafts with syngeneic epithelium presented in the DLN is probably insufficient for sensitizing recipients and for mounting an effective rejection response even when placed in high-risk corneal beds.[52]

All in all, this suggests that both pathways operate in corneal allotransplantation but only when the number of corneal LC present in DLN is artificially enhanced (either by increasing their number in grafts or if neovascularization/lymphangiogenesis is present), does the direct mode of allorecognition significantly contribute.

THE IMMUNE RESPONSE - EFFERENT ARM

Innate immunity is involved from the moment of transplantation. It is induced by the tissue trauma and the following wound healing and works in a close cooperation with corneal cells. Its main activity lasts several days after transplantation (as we know from syngeneic transplantations); later its activity ceases. Clinically, after several days the anterior segment becomes relatively quiet – until the onset of rejection.

Corneal wound healing is a very complex reaction involving reciprocal interactions of corneal as well as inflammatory cells and is mediated by cytokines, extracellular matrix components and intercellular contacts, as reviewed by Wilson *et al.*).[72] Transparency of the optical axis is crucial for good vision and several mechanisms have evolved for its protection. A number of extracellular matrix components act in an immunomodulatory fashion. For instance, the cornea contains heparan sulfate, which is capable of inhibiting release of some pro-angiogenic factors such as basic fibroblast growth factor. In addition, during degradation of corneal extracellular matrix, a number of potent angiostatic components, e.g. angiostatin and endostatin, are produced.

The body also contains various stem cells, which are able to differentiate into any mature cell type. Recently, CD34+ hemopoietic stem cells have been demonstrated in

the mouse corneal stroma;[58] however, their role in wound healing or tissue regeneration must be studied further.

There are two routes by which inflammatory cells can access the graft - either through the recipient cornea or through the anterior chamber after the disruption of the blood-ocular barrier. In syngeneic transplantation, during the first week grafts are infiltrated with a mixture of macrophages and CD4+T cells (2:1 ratio) with their number promptly decreasing after one or two weeks. By the fourth week, they persist in the wound area but not in the center of the graft.[73] In allografts, the earliest arriving cells, within 15 minutes after transplantation, are CD11b+MOMA-2+F4/80+ myeloid cells and granulocytes at the limbus and by two hours at the graft interface (plus some F4/80+ cells and neutrophils detected in the graft). The first CD8+ T cells appear in the graft after 16 hours in contrast to CD4+ cells which need two days. In syngeneic transplantation, the early kinetics is similar but with a slower pace. In allografts, the tissue is infiltrated during rejection by numerous macrophages, T cells (again mainly CD4+), NK and neutrophils.[74] The ratio is reversed after three to four weeks post-grafting when CD8+T cells predominate over CD4+ cells.[73,74] NK cells were detected during rejection in the epithelial rejection line, the corneal stroma as well as AH[73,74] and their role is especially dominant in immature recipients.

In high-risk transplantations, grafts are intensively infiltrated especially by macrophages and neutrophils and to some extent also by mast cells with smaller number of mast cells and eosinophils detected also in normal-risk allografts.[75]

In AH, CD4+T cells and cells of monocyte/macrophage origin predominate two days after transplantation and the number of T and NK cells further increase later on.[76]

Within the first three days of rejection CD8+ cells predominate over CD4+ cells, while 5-8 days after the beginning of rejection this ratio is reversed.[77]

Several studies have evaluated DLN response after transplantation; however, these results are difficult to interpret because the percentage of cell populations rather than the total cell numbers were analyzed. The crucial role of DLN in graft rejection and prevention of rejection was shown in both normal- and high-risk models of corneal transplantation,[78-80] as well as in other models earlier.[81,82] Donor-specific cytotoxic T cells were detected and their appearance coincided with graft rejection only in a neovascularized high-risk model.[83]

The direct depletion/inhibition of individual cell populations may indicate their role, importance or substitutability in the transplant reaction. Under lymphopenic conditions, we may have to take into account the homeostatic proliferation of lymphocytes (fox example of CD4+CD25+ Tregs);[84] however, this concept has not been tested in corneal transplantation models yet.

CD4+T cells are considered the main effector cells in the indirect allorecognition. Their contribution was experimentally verified by Hašková *et al.* who demonstrated that in normal-risk recipients CD4+ cells are necessary for the corneal graft rejection, but not for rejection of skin grafts.[85] Similar results were obtained by Yamada *et al.* and in high-risk corneal recipients by Vítová *et al.*.[86,87] Their precise role in rejection is still insufficiently understood. They may operate as helper cells in the generation of allospecific effector macrophages (with subsequent release of a variety of soluble mediators) as well as apoptosis-inducing effector cells.

CD8+T lymphocytes are recognized as central to the direct allorecognition but their role in corneal transplantation is not yet completely understood. From several reports, we know that they contribute to the process of rejection but they do not seem to be crucial.[85,88] CD8+T cell-mediated cytotoxicity involves local secretion of cytotoxic cytokines (TNF-α and IFN-γ) and apoptosis-inducing mechanisms such as perforin/granzyme, or Fas/FasL pathway. Apoptosis of keratocytes has been detected in rejecting corneal grafts (with higher intensity after high-risk transplantation) and it could be induced *in vitro* by combined stimulation with agonistic Fas monoclonal antibody (mAb) and either IL-1β or TNF-α.[89] Another potential effector molecule may be granulysin (a cationic molecule of CD8+ cytotoxic T lymphocytes (CTL), CD4+CTL and NK cells) upregulation of which was detected during acute renal rejection.[90]

$\gamma\delta$ T cells represent a small subset of T cells (usually CD4-CD8- but may be also CD8+) and are often associated with various forms of tolerance induction in other systems but also with ACAID. $\gamma\delta$ T cells are required for the generation of CD8+ suppressor cells and their production of IL-10 seems to be dominant in the generation of ACAID Tregs.[91] The blockage of T cell receptor-delta chain and their functional inhibition results in the shorter corneal allograft survival.[92]

Similarly, through an IL-10-dependent regulatory mechanism, the population of NKT cells is critical for the development of antigen-specific Tregs in ACAID. In NKT knock out (KO) animals, rejection rate is 100% in contrast to 50% in wild type animals.[93] In model of skin transplantation, NKT activation requires CD1d+ host APC and the migration of NKT cells into grafts.[94]

The role of the humoral immune response and the antibody-mediated damage in corneal transplantation remains controversial. The role of B cells is in this transplantation model normally considered secondary to T cell responses. B cells are recognized as antibody producing cells, "contributing" to the tissue injury through complement-mediated reactions. Accumulation of antibodies in both allografts and xenografts has been demonstrated,[95] but rejection of xenografts in mouse deficient in the μ heavy chain of immunoglobulin gene is not delayed.[96] In contrast, experiments in corneal allografts using B cell KO or deficient mice[97] showed that, although antibodies were not necessary for corneal rejection, their presence could produce an additional extensive injury in a complement-dependent manner. However, in both allo- and xenotransplantation models corneal grafts are vulnerable to antibody-mediated rejection when recipients are treated with donor-specific antisera.[98]

The cells of monocyte/macrophage origin are recruited to the graft in large numbers. The role of T cells is critical but allograft rejection occurs also in situations when Fas/FasL, perforin or TNF-α pathways are blocked. CD4+T cells function as helper cells in the generation of allospecific effector macrophages.[99] During the early phase after transplantation, activated macrophages (in addition to neutrophils) contribute to the innate arm of the immune response and the tissue healing by removal of the dead tissue and by the production of growth factors stimulating fibroblast proliferation, collagen synthesis and angiogenesis. During later stages of rejection, they likely contribute as effector cells in the acquired arm of the immune response. The full extent of this contribution is unknown. After activation, macrophages, in response to CD40 signals and IFN-γ, produce various reactive oxygen intermediates, nitric oxide (NO), and lysozomal enzymes. The activation of the inducible form of nitric oxide synthase (iNOS) and the subsequent production of free NO radicals play an important role in

anti-infectious and anti-tumor immunity as reviewed by Korhonen e*t al.*[100]. During acute allograft rejection activated macrophages expressing a T cell-dependently iNOS[101] were detected.[102] These sites were characterized by a large degree of tissue damage and apoptotic cells. In a mouse model of corneal transplantation, treatment with aminoguanidine, a specific inhibitor of iNOS, significantly delayed allograft rejection.[103] Macrophage depletion or their inactivation may limit the extent of rejection by the reduction of the tissue injury. Subconjunctival injection of dichlormethylene diphosphonate encapsulated in liposomes prevented corneal allograft rejection through depletion of macrophages but this treatment affected also the afferent arm of the immune response by yet unexplained mechanism.[104]

CLINICAL FORMS OF HUMAN ALLOGRAFT REJECTION

In humans, we recognize four clinical forms: epithelial, subepithelial infiltrates, stromal and endothelial rejection.

Epithelial rejection is visible as a fine, whitish line beginning in the graft periphery and crossing centrally over several days to weeks. It consists of polymorphonuclear cells with lymphocytes (some of them in blast form) and destroys epithelial cells which are replaced by cells of recipient origin.[105] Although the process is usually self-limiting, it is a sign of higher immunological activity and it may progress to an endothelial rejection.

Subepithelial infiltrates appear as multiple discrete white infiltrates in otherwise quiet eyes. This usually happens later than in the case of epithelial rejection. The infiltrates are located immediately underneath the epithelium in the anterior stroma, and their composition is unknown. They can be associated with endothelial or epithelial rejection

and usually promptly disappear after treatment with topical steroids, although they can leave a faint scar. Similar to epithelial rejection, infiltrates are a sign of a low-grade, chronic immunologic reaction.

Stromal rejection is sometimes distinguished as a separate entity although it is often associated with endothelial rejection, which makes clear diagnosis difficult. Clinically, the sudden onset of circumlimbal injection, edema, and stromal haze near vessels in the otherwise clear cornea which starts at the periphery and moves toward the centre is present. Histologically, it correlates with the infiltration of monocytes, T, and B lymphocytes.

Transparency of the corneal graft depends on the integrity of the endothelial cells. Two main forms of endothelial rejection can be distinguished according to the morphological appearance. The local form of rejection is characterized by fibrin and the appearance of the Khodadoust line formed by the aggregation of lymphocytes adherent to the endothelial surface. In addition, we can see keratic precipitates with or without the Khodadoust line, which can be responsible for a severe cell loss.

The second form - more slowly, but steadily occurring - is the diffuse form. It is responsible for a gradual decrease in endothelial cell density. Chronic immune mechanisms may contribute to this decrease because the extent of cell loss directly depends on HLA incompatibility[106] with contribution of other factors such as corneal preservation, or cadaver time. Several effector mechanisms including Fas/FasL interaction, perforin and granzyme release, and reactive oxygen species are involved. In addition, proinflammatory cytokines such as TNF-α, IFN-γ, and IL-1 by their

synergistic effect may mediate apoptosis through the induction of iNOS. Of the cytokines mentioned, TNF-α is capable of directly inducing cellular apoptosis.

PERSISTENCE OF GRAFT CELLS AFTER CORNEAL TRANSPLANTATION

Initially, it was believed that the transplant eventually disappears and that its main role is to operate as a framework for recipient's cells. This is not entirely true. The replacement of corneal epithelium in mice after both syngeneic as well allogeneic transplantation occurred in 15 days (several days before beginning of rejection was clinically apparent). The authors concluded that epithelium is not a target of alloimmune rejection and therefore mouse allograft models are not a good model for studying corneal epithelial rejection.[107] In contrast to allografts in mice, the severely inflamed immunologically rejected xenografts do not support host-derived reepithelization. However, when the inflammatory response is sufficiently suppressed, donor graft epithelial coverage by host cells is permitted.[108] In rabbit and rat, the rejection of allograft epithelium is characterized by a linear defect consisting of dying epithelial cells and inflammatory cells. If allografts are accepted, donor epithelium may stay for a long period.

Donor keratocytes persist in accepted mouse corneal allografts (similarly to cat syngeneic grafts) to a high level[109] but not in grafts which were rejected. Apoptosis of keratocytes *in vitro* can be induced by combined stimulation with agonistic Fas mAb and either IL-1β or TNF-α and is more prominent after high-risk transplantation.

Corneal endothelium has a crucial role in the maintenance of corneal transparency. For a long time it was thought that in human, rat and mouse (but not in rabbit) corneal endothelium did not proliferate and when damaged the loss appeared to be final.

However, a recent report suggests that stem-like cells located in the posterior limbus may be a source of renewed endothelial cells similar to the epithelium.[110] Corneal endothelium (similarly to keratocytes) persists to a high degree in syngeneic[111] and accepted allografts, but it is destroyed and removed during rejection. In addition, it is known that the endothelial cell density decreases with time after surgery and chronic immune mechanisms may be involved.[106]

XENOTRANSPLANTATION

Despite the fact that corneal grafts are the most frequently transplanted tissue,[2] there is a continuing shortage of the donor material. Accordingly, corneal xenografts have long been considered a possibility as early as 1838 (R. Kissam).[112]

Xenografts are characterized by a greater antigenic difference that exist between different species, and therefore the immune response to xenografts is much stronger than to allografts and is difficult to overcome. In concordant xenograft transplantation, tissue is transplanted between closely related species, while in discordant xenografts between more disparate species.

Porcine corneas are for many reasons believed to be the most suitable possible source of xenografts in the future. The majority of human anti-pig natural antibodies are directed against one particular carbohydrate determinant - α-galactoside. There is some experimental evidence that porcine corneas lack alpha-galactoside epitope (except for several keratocytes in the most anterior part).[113,114] Yamagami *et al.* showed that the corneal concordant xenotransplantation model is characterized by a delayed onset of rejection (opaque grafts at day 6), with strong cellular infiltration only

in the recipient cornea (mainly by T cells with contribution of antibody dependent cytotoxicity) and only later on to some extent in grafts.[115]

The mechanisms responsible for xenograft rejection have been reviewed by Qian *et al.* in corneal transplants in experimental animals.[116] The immunoprivileged environment of the cornea appears to provide corneal xenogeneic grafts with some degree of protection, as hyperacute or acute vascular rejection as described for other solid organ transplants does not occur here.[115] The rejection of corneal transplants is a slower process which is, however, associated with severe inflammation throughout the entire anterior segment, with necrosis at the graft margin and sloughing of the xenograft. Under conditions when the inflammatory response is attenuated, graft infiltration at the late time point is possibly enabled.[108]

Corneal xenograft rejection is mediated by CD4+T cells with a minor contribution from complement.[96] CD8+T cells and NKT cells are not obligatory, but may play a role in rejection in situations where CD4+T cells are absent or their function is impaired.[117,118] CD8+T cells were not able to lyse target guinea pig cells *in vitro*, but produced a significant amount of IFN-γ that may mediate this effect.[118] In addition, recently Tanaka *et al.* showed that eotaxin (a potent eosinophil chemoattractant) is overexpressed during corneal xenograft rejection, and eosinophils represent the majority of infiltrating granulocytes.[119]

The role of the humoral immune response in corneal xenograft rejection was outlined in the text above. Xenograft rejection is associated with the upregulation of the T_H1 type cytokine expression (IL-2 and IFN-γ) initially in the recipient cornea and later in the graft[115] and with increased NO production. T_H2 type cytokines are upregulated but

do not correlate with rejection. Treatment of recipients with anti-CD4 mAb intensely inhibits IL-2, IL-4 and IL-10 production and promotes corneal xenograft survival.[120]

CONCLUSION

Long years of evolution have provided the cornea with the advantage of the immune privilege but we should always bear in mind its limits. Firstly, factors involved and the relationships that exist between them have to be recognized; secondly, we need to learn how to use those factors for our benefit. When thinking about the immune mechanisms, it is necessary to realize that although the immune reaction starts in the eye, an important part is also played outside – in draining lymph nodes, the spleen and the thymus.

Despite the already acquired knowledge in this field, further research is needed to better understand the subtle mechanisms operating within (and outside) this niche and to improve the prospect of corneal grafts.

ACKNOWLEDGMENT

The authors would like to thank Assoc. Prof. Vladimir Holáň (Institute of Molecular Genetics, Academy of Science of the Czech Republic, Prague, Czech Republic) for critical reading of the manuscript.

REFERENCES

1. Williams KA, Esterman AJ, Bartlett C, Holland H, Hornsby NB, Coster DJ. How effective is penetrating corneal transplantation? Factors influencing long-term outcome in multivariate analysis. *Transplantation* 2006; **81**: 896-901.
2. The collaborative corneal transplantation studies (CCTS). Effectiveness of histocompatibility matching in high-risk corneal transplantation. The Collaborative Corneal Transplantation Studies Research Group. *Arch Ophthalmol* 1992; **110**: 1392-403.
3. Streilein J, Stein-Streilein J. Does innate immune privilege exist? *J Leukoc Biol* 2000; **67**: 479-87.
4. Cursiefen C, Maruyama K, Jackson DG, Streilein JW, Kruse FE. Time course of angiogenesis and lymphangiogenesis after brief corneal inflammation. *Cornea* 2006; **25**: 443-7.
5. Stuart PM, Yin X, Plambeck S, Pan F, Ferguson TA. The role of Fas ligand as an effector molecule in corneal graft rejection. *Eur J Immunol* 2005; **35**: 2591-7.
6. Chen J-J, Sun Y, Nabel GJ. Regulation of the proinflammatory effects of Fas ligand (CD95L). *Science* 1998; **282**: 1714-17.
7. Lee H-o, Herndon JM, Barreiro R, Griffith TS, Ferguson TA. TRAIL: A Mechanism of Tumor Surveillance in an Immune Privileged Site. *J Immunol* 2002; **169**: 4739-44.
8. Hori J, Wang M, Miyashita M *et al.* B7-H1-induced apoptosis as a mechanism of immune privilege of corneal allografts. *J Immunol* 2006; **177**: 5928-35.
9. Donnelly JJ, Orlin SE, Wei ZG, Raber IM, Rockey JH. Class II alloantigen induced on corneal endothelium. Role in corneal allograft rejection. *Invest Ophthalmol Vis Sci* 1990; **31**: 1315-20.

10. Figueiredo FC, Pendergrast DG, Zhang L, Easty DL, Nicholls SM. An improved method for examining the corneal endothelium during graft rejection in the rat. *Exp Eye Res* 1998; **67**: 625-30.

11. Niederkorn JY, Chiang EY, Ungchusri T, Stroynowski I. Expression of a nonclassical MHC class Ib molecule in the eye. *Transplantation* 1999; **68**: 1790-9.

12. Chiang EY, Henson M, Stroynowski I. The nonclassical major histocompatibility complex molecule Qa-2 protects tumor cells from NK cell- and lymphokine-activated killer cell-mediated cytolysis. *J Immunol* 2002; **168**: 2200-11.

13. D'Orazio TJ, Niederkorn JY. A novel role for TGF-beta and IL-10 in the induction of immune privilege. *J Immunol* 1998; **160**: 2089-98.

14. Wang Y, Goldschneider I, O'Rourke J, Cone RE. Blood mononuclear cells induce regulatory NK T thymocytes in anterior chamber-associated immune deviation. *J Leukoc Biol* 2001; **69**: 741-6.

15. Wang Y, Goldschneider I, Foss D, Wu DY, O'Rourke J, Cone RE. Direct thymic involvement in anterior chamber-associated immune deviation: evidence for a nondeletional mechanism of centrally induced tolerance to extrathymic antigens in adult mice. *J Immunol* 1997; **158**: 2150-5.

16. Li X, Wang Y, Urso D, O'Rourke J, Cone RE. Thymocytes induced by antigen injection into the anterior chamber activate splenic CD8+ suppressor cells and enhance the antigen-induced production of immunoglobulin G1 antibodies. *Immunology* 2004; **113**: 44-56.

17. Ashour HM, Niederkorn JY. Peripheral tolerance via the anterior chamber of the eye: Role of B cells in MHC class I and II antigen presentation. *J Immunol* 2006; **176**: 5950-57.

18. Wilbanks G, Mammolenti M, Streilein J. Studies on the induction of anterior chamber-associated immune deviation (ACAID). II. Eye-derived cells participate in generating blood-borne signals that induce ACAID. *J Immunol* 1991; **146**: 3018-24.

19. Dullforce PA, Garman KL, Seitz GW *et al.* APCs in the anterior uveal tract do not migrate to draining lymph nodes. *J Immunol* 2004; **172**: 6701-08.

20. Wilbanks GA, Streilein JW. Fluids from immune privileged sites endow macrophages with the capacity to induce antigen-specific immune deviation via a mechanism involving transforming growth factor-beta. *Eur J Immunol* 1992; **22**: 1031-6.

21. Wilbanks GA, Streilein JW. Distinctive humoral immune responses following anterior chamber and intravenous administration of soluble antigen. Evidence for active suppression of IgG2-secreting B lymphocytes. *Immunology* 1990; **71**: 566-72.

22. Hara Y, Caspi R, Wiggert B, Dorf M, Streilein J. Analysis of an in vitro-generated signal that induces systemic immune deviation similar to that elicited by antigen injected into the anterior chamber of the eye [published erratum appears in J Immunol 1992 Dec 15;149(12):4116]. *J Immunol* 1992; **149**: 1531-38.

23. Taylor A. A review of the influence of aqueous humor on immunity. *Ocul Immunol Inflamm* 2003; **11**: 231-41.

24. Streilein JW, Okamoto S, Sano Y, Taylor AW. Neural control of ocular immune privilege. *Ann NY Acad Sci* 2000; **917**: 297-306.

25. Apte RS, Sinha D, Mayhew E, Wistow GJ, Niederkorn JY. Cutting edge: Role of macrophage migration inhibitoryfactor in inhibiting NK cell activity and preserving immune privilege. *J Immunol* 1998; **160**: 5693-96.

26. Sohn J-H, Kaplan HJ, Suk H-J, Bora PS, Bora NS. Complement regulatory activity of normal human intraocular fluid is mediated by MCP, DAF, and CD59. *Invest Ophthalmol Vis Sci* 2000; **41**: 4195-202.

27. Treseler PA, Foulks GN, Sanfilippo F. The expression of HLA antigens by cells in the human cornea. *Am J Ophthalmol* 1984; **98**: 763-72.

28. Jager MJ. Corneal Langerhans cells and ocular immunology. *Reg Immunol* 1992; **4**: 186-95.

29. Young E, Stark WJ, Prendergast RA. Immunology of corneal allograft rejection: HLA-DR antigens on human corneal cells. *Invest Ophthalmol Vis Sci* 1985; **26**: 571-4.

30. Arancibia-Carcamo CV, Osawa H, Arnett HA *et al.* A CIITA-independent pathway that promotes expression of endogenous rather than exogenous peptides in immune-privileged sites. *Eur J Immunol* 2004; **34**: 471-80.

31. Khaireddin R, Wachtlin J, Hopfenmuller W, Hoffmann F. HLA-A, HLA-B and HLA-DR matching reduces the rate of corneal allograft rejection. *Graefes Arch Clin Exp Ophthalmol* 2003; **241**: 1020-8.

32. Reinhard T, Bohringer D, Enczmann J *et al.* Improvement of graft prognosis in penetrating normal-risk keratoplasty by HLA class I and II matching. *Eye* 2004; **18**: 269-77.

33. Wachtlin J, Khaireddin R, Hoffmann F. [Tissue typing in perforating corneal transplantation]. *Ophthalmologe* 2003; **100**: 1021-30.

34. Hopkins KA, Maguire MG, Fink NE, Bias WB. Reproducibility of HLA-A, B, and DR typing using peripheral blood samples: results of retyping in the collaborative corneal transplantation studies. Collaborative corneal transplantation studies group (corrected). *Hum Immunol* 1992; **33**: 122-8.

35. Lloyd KO. The chemistry and immunochemistry of blood group A, B, H, and Lewis antigens: past, present and future. *Glycoconj J* 2000; **17**: 531-41.

36. Salisbury JD, Gebhardt BM. Blood group antigens on human corneal cells demonstrated by immunoperoxidase staining. *Am J Ophthalmol* 1981; **91**: 46-50.

37. Pedal I, Wetzel C, Min JX, Daus W. ABH and Lewis immunohistochemistry of the human eye. *Forensic Sci Int* 1995; **71**: 51-5.

38. Dua HS, Shidham VB. Application of specific red blood cell adherence test to the human cornea and conjunctiva. *Am J Ophthalmol* 1979; **88**: 1067-71.

39. Foster CS, Allansmith MR. Lack of blood group antigen A on human corneal endothelium. *Am J Ophthalmol* 1979; **87**: 165-70.

40. Volker-Dieben HJ, Claas FH, Schreuder GM *et al.* Beneficial effect of HLA-DR matching on the survival of corneal allografts. *Transplantation* 2000; **70**: 640-8.

41. Roy R, Des Marchais B, Bazin R, Boisjoly HM, Dube I, Laughrea PA. Role of ABO and Lewis blood group antigens in donor-recipient compatibility of corneal transplantation rejection. *Ophthalmology* 1997; **104**: 508-12.

42. Volker-Dieben HJ, Kok-van Alphen CC, Lansbergen Q, Persijn GG. Different influences on corneal graft survival in 539 transplants. *Acta Ophthalmol (Copenh)* 1982; **60**: 190-202.

43. Chan JH, Dua HS, Powell-Richards A, Jones DR, Harris IM. Effect of ABO blood group mismatching on corneal epithelial cells: an in vitro study. *Br J Ophthalmol* 2001; **85**: 1104-9.

44. Perreault C, Decary F, Brochu S, Gyger M, Belanger R, Roy D. Minor histocompatibility antigens. *Blood* 1990; **76**: 1269-80.

45. Sullivan K, Shulman H, Storb R *et al.* Chronic graft-versus-host disease in 52 patients: adverse natural course and successful treatment with combination immunosuppression. *Blood* 1981; **57**: 267-76.

46. Bailey DW, Mobraaten LE. Estimates of the number of loci contributing to the histoincompatibility between C57BL-6 and BALB-c strains of mice. *Transplantation* 1969; **7**: 394-400.

47. Joo CK, Pepose JS, Stuart PM. T-cell mediated responses in a murine model of orthotopic corneal transplantation. *Invest Ophthalmol Vis Sci* 1995; **36**: 1530-40.

48. Haskova Z, Sproule TJ, Roopenian DC, Ksander AB. An immunodominant minor histocompatibility alloantigen that initiates corneal allograft rejection. *Transplantation* 2003; **75**: 1368-74.

49. Haskova Z, Filipec M, Holan V. The role of major and minor histocompatibility antigens in orthotopic corneal transplantation in mice. *Folia Biol (Praha)* 1996; **42**: 105-11.

50. Bohringer D, Spierings E, Enczmann J *et al.* Matching of the minor histocompatibility antigen HLA-A1/H-Y may improve prognosis in corneal transplantation. *Transplantation* 2006; **82**: 1037-41.

51. Hamrah P, Zhang Q, Liu Y, Dana MR. Novel characterization of MHC class II-negative population of resident corneal Langerhans cell-type dendritic cells. *Invest Ophthalmol Vis Sci* 2002; **43**: 639-46.

52. Hori J, Streilein JW. Survival in high-risk eyes of epithelium-deprived orthotopic corneal allografts reconstituted in vitro with syngeneic epithelium. *Invest Ophthalmol Vis Sci* 2003; **44**: 658-64.

53. Hori J, Streilein JW. Role of recipient epithelium inpromoting survival of orthotopic corneal allografts in mice. *Invest Ophthalmol Vis Sci* 2001; **42**: 720-26.

54. Ardjomand N, Berghold A, Komericki P, Reich ME. [The significance of tissue storage time for success after corneal transplantation]. *Ophthalmologe* 1997; 94: 285-9.

55. Holland EJ, DeRuyter DN, Doughman DJ. Langerhans cells in organ-cultured corneas. *Arch Ophthalmol* 1987; **105**: 542-5.

56. Hamrah P, Liu Y, Zhang Q, Dana MR. The corneal stroma is endowed with a significant number of resident dendritic cells. *Invest Ophthalmol Vis Sci* 2003; **44**: 581-9.

57. Brissette-Storkus CS, Reynolds SM, Lepisto AJ, Hendricks RL. Identification of a novel macrophage population in the normal mouse corneal stroma. *Invest Ophthalmol Vis Sci* 2002; **43**: 2264-71.

58. Sosnova M, Bradl M, Forrester JV. CD34+ corneal stromal cells are bone marrow-derived and express hemopoietic stem cell markers. *Stem Cells* 2005; **23**: 507-15.

59. Hamrah P, Liu Y, Zhang Q, Dana MR. Alterations in corneal stromal dendritic cell phenotype and distribution in inflammation. *Arch Ophthalmol* 2003; **121**: 1132-40.

60. Becker MD, Planck SR, Crespo S *et al.* Immunohistology of antigen-presenting cells in vivo: a novel method for serial observation of fluorescently labeled cells. *Invest Ophthalmol Vis Sci* 2003; **44**: 2004-9.

61. McMenamin PG. Dendritic cells and macrophages in the uveal tract of the normal mouse eye. *Br J Ophthalmol* 1999; **83**: 598-604.

62. McMenamin PG, Crewe J, Morrison S, Holt PG. Immunomorphologic studies of macrophages and MHC class II-positive dendritic cells in the iris and ciliary body of the rat, mouse, and human eye. *Invest Ophthalmol Vis Sci* 1994; **35**: 3234-50.

63. Camelo S, Kezic J, Shanley A, Rigby P, McMenamin PG. Antigen from the anterior chamber of the eye travels in a soluble form to secondary lymphoid organs via lymphatic and vascular routes. *Invest Ophthalmol Vis Sci* 2006; **47**: 1039-46.

64. Camelo S, Shanley A, Voon ASP, McMenamin PG. The distribution of antigen in lymphoid tissues following its injection into the anterior chamber of the rat eye. *J Immunol* 2004; **172**: 5388-95.

65. Kaplan HJ, Streilein JW. Do immunologically privileged sites require a functioning spleen? *Nature* 1974; **251**: 553-4.

66. Liu Y, Hamrah P, Zhang Q, Taylor AW, Dana MR. Draining lymph nodes of corneal transplant hosts exhibit evidence for donor major histocompatibility complex (MHC) class II-positive dendritic cells derived from MHC class II-negative grafts. *J Exp Med* 2002; **195**: 259-68.

67. Hoffmann F, Zhang EP, Mueller A *et al.* Contribution of lymphatic drainage system in corneal allograft rejection in mice. *Graefes Arch Clin Exp Ophthalmol* 2001; **239**: 850-8.

68. Collin HB. Lymphatic drainage of 131I-albumin from the vascularized cornea. *Invest Ophthalmol Vis Sci* 1970; **9**: 146-55.

69. Itano AA, McSorley SJ, Reinhardt RL *et al.* Distinct dendritic cell populations sequentially present antigen to CD4 T cells and stimulate different aspects of cell-mediated immunity. *Immunity* 2003; **19**: 47-57.

70. Boisgerault F, Liu Y, Anosova N, Ehrlich E, Dana MR, Benichou G. Role of CD4+ and CD8+ T cells in allorecognition: lessons from corneal transplantation. *J Immunol* 2001; **167**: 1891-9.

71. Huq S, Liu Y, Benichou G, Dana MR. Relevance of the direct pathway of sensitization in corneal transplantation is dictated by the graft bed microenvironment. *J Immunol* 2004; **173**: 4464-9.

72. Wilson SE, Mohan RR, Ambrosio R, Jr., Hong J, Lee J. The corneal wound healing response: cytokine-mediated interaction of the epithelium, stroma, and inflammatory cells. *Prog Retin Eye Res* 2001; **20**: 625-37.

73. Holland EJ, Olsen TW, Chan CC, Bergstrom L, Palestine AG, Nussenblatt RB. Kinetics of corneal transplant rejection in the rat penetrating keratoplasty model. *Cornea* 1994; **13**: 317-23.

74. Kuffova L, Lumsden L, Vesela V *et al.* Kinetics of leukocyte and myeloid cell traffic in the murine corneal allograft response. *Transplantation* 2001; **72**: 1292-8.

75. Yamagami S, Hamrah P, Zhang Q, Liu Y, Huq S, Dana MR. Early ocular chemokine gene expression and leukocyte infiltration after high-risk corneal transplantation. *Mol Vis* 2005; **11**: 632-40.

76. Claerhout I, Kestelyn P, Debacker V, Beele H, Leclercq G. Role of natural killer cells in the rejection process of corneal allografts in rats. *Transplantation* 2004; **77**: 676-82.

77. Larkin DF, Calder VL, Lightman SL. Identification and characterization of cells infiltrating the graft and aqueous humor in rat corneal allograft rejection. *Clin Exp Immunol* 1997; **107**: 381-91.

78. Plskova J, Duncan L, Holan V, Filipec M, Kraal G, Forrester JV. The immune response to corneal allograft requires a site-specific draining lymph node. *Transplantation* 2002; **73**: 210-5.

79. Yamagami S, Dana MR, Tsuru T. Draining lymph nodes play an essential role in alloimmunity generated in response to high-risk corneal transplantation. *Cornea* 2002; **21**: 405-9.

80. Yamagami S, Dana MR. The critical role of lymph nodes in corneal alloimmunization and graft rejection. *Invest Ophthalmol Vis Sci* 2001; **42**: 1293-8.

81. Lakkis FG, Arakelov A, Konieczny BT, Inoue Y. Immunologic 'ignorance' of vascularized organ transplants in the absence of secondary lymphoid tissue. *Nat Med* 2000; **6**: 686-8.

82. Barker CF, Billingham RE. The role of afferent lymphatics in the rejection of skin homografts. *J Exp Med* 1968; **128**: 197-221.

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

84. Graca L, Chen TC, Le Moine A, Cobbold SP, Howie D, Waldmann H. Dominant tolerance: activation thresholds for peripheral generation of regulatory T cells. *Trends Immunol* 2005; **26**: 130-5.

85. Haskova Z, Usiu N, Pepose JS, Ferguson TA, Stuart PM. CD4+ T cells are critical for corneal, but not skin, allograft rejection. *Transplantation* 2000; **69**: 483-7.

86. Yamada J, Kurimoto I, Streilein JW. Role of CD4+ T Cells in Immunobiology of Orthotopic Corneal Transplants in Mice. *Invest Ophthalmol Vis Sci* 1999; **40**: 2614-21.

87. Vitova A, Filipec M, Zajicova A, Krulova M, Holan V. Prevention of corneal allograft rejection in a mouse model of high risk recipients. *Br J Ophthalmol* 2004; **88**: 1338-42.

88. Niederkorn JY, Stevens C, Mellon J, Mayhew E. CD4+ T-cell-independent rejection of corneal allografts. *Transplantation* 2006; **81**: 1171-8.

89. Beauregard C, Huq SO, Barabino S, Zhang Q, Kazlauskas A, Dana MR. Keratocyte apoptosis and failure of corneal allografts. *Transplantation* 2006; **81**: 1577-82.

90. Krensky AM, Clayberger C. Granulysin: a novel host defense molecule. *Am J Transplant* 2005; **5**: 1789-92.

91. Ashour HM, Niederkorn JY. Gammadelta T cells promote anterior chamber-associated immune deviation and immune privilege through their production of IL-10. *J Immunol* 2006; **177**: 8331-7.

92. Skelsey ME, Mellon J, Niederkorn JY. Gamma delta T cells are needed for ocular immune privilege and corneal graft survival. *J Immunol* 2001; **166**: 4327-33.

93. Sonoda K-H, Taniguchi M, Stein-Streilein J. Long-term survival of corneal allografts is sependent on intact CD1d-reactive NKT cells. *J Immunol* 2002; **168**: 2028-34.

94. Oh K, Kim S, Park S-H *et al.* Direct regulatory role of NKT cells in allogeneic graft survival is dependent on the quantitative strength of antigenicity. *J Immunol* 2005; **174**: 2030-36.

95. Larkin DF, Takano T, Standfield SD, Williams KA. Experimental orthotopic corneal xenotransplantation in the rat. Mechanisms of graft rejection. *Transplantation* 1995; **60**: 491-7.

96. Tanaka K, Yamada J, Streilein JW. Xenoreactive CD4+ T cells and acute rejection of orthotopic guinea pig corneas in mice. *Invest Ophthalmol Vis Sci* 2000; **41**: 1827-32.

97. Hegde S, Mellon JK, Hargrave SL, Niederkorn JY. Effect of alloantibodies on corneal allograft survival. *Invest Ophthalmol Vis Sci* 2002; **43**: 1012-8.

98. Holan V, Vitova A, Krulova M *et al.* Susceptibility of corneal allografts and xenografts to antibody-mediated rejection. *Immunol Lett* 2005; **100**: 211-3.

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

100. Korhonen R, Lahti A, Kankaanranta H, Moilanen E. Nitric oxide production and signaling in inflammation. *Curr Drug Targets Inflamm Allergy* 2005; **4**: 471-9.

101. Langrehr JM, White DA, Hoffman RA, Simmons RL. Macrophages produce nitric oxide at allograft sites. *Ann Surg* 1993; **218**: 159-66.

102. Krulova M, Zajicova A, Fric J, Holan V. Alloantigen-induced, T-cell-dependent production of nitric oxide by macrophages infiltrating skin allografts in mice. *Transpl Int* 2002; **15**: 108-16.

103. Strestikova P, Plskova J, Filipec M, Farghali H. FK 506 and aminoguanidine suppress iNOS induction in orthotopic corneal allografts and prolong graft survival in mice. *Nitric Oxide* 2003; **9**: 111-7.

104. Slegers TP, Broersma L, van Rooijen N, Hooymans JM, van Rij G, van der Gaag R. Macrophages play a role in the early phase of corneal allograft rejection in rats. *Transplantation* 2004; **77**: 1641-6.

105. Kanai A, Polack FM. Ultramicroscopic alterations in corneal epithelium in corneal grafts. *Am J Ophthalmol* 1971; **72**: 119-26.

106. Ruusuvaara P. Histocompatibility and corneal graft endothelium. *Acta Ophthalmol (Copenh)* 1979; **57**: 968-81.

107. Hori J, Streilein JW. Dynamics of donor cell persistence and recipient cell replacement in orthotopic corneal allografts in mice. *Invest Ophthalmol Vis Sci* 2001; **42**: 1820-28.

108. Sedlakova K, Muckersie E, Robertson M, Filipec M, Forrester JV. FTY720 in corneal concordant xenotransplantation. *Transplantation* 2005; **79**: 297-303.

109. Basu PK, Miller I, Ormsby HL. Sex chromatin as a biologic cell marker in the study of the fate of corneal transplants. *Am J Ophthalmol* 1960; **49**: 513-5.

110. Whikehart DR, Parikh CH, Vaughn AV, Mishler K, Edelhauser HF. Evidence suggesting the existence of stem cells for the human corneal endothelium. *Mol Vis* 2005; **11**: 816-24.

111. Chi HH, Teng CC, Katzin HM. The fate of endothelial cells in corneal homografts. *Am J Ophthalmol* 1965; **59**: 186-91.

112. Kissam RS. Ceratoplastice in man. *New York J Med* 1844; **2**: 281-82.

113. Amano S, Shimomura N, Kaji Y, Ishii K, Yamagami S, Araie M. Antigenicity of porcine cornea as xenograft. *Curr Eye Res* 2003; **26**: 313-8.

114. Hrdlickova-Cela E, Smetana K, Jr., Plzak J *et al.* Cells of porcine epidermis and corneal epithelium are not recognized by human natural anti-alpha-galactoside IgG. *Folia Biol (Praha)* 2001; **47**: 200-5.

115. Yamagami S, Isobe M, Yamagami H, Hori J, Tsuru T. Mechanism of concordant corneal xenograft rejection in mice: synergistic effects of anti-leukocyte function-associated antigen-1 monoclonal antibody and FK506. *Transplantation* 1997; **64**: 42-8.

116. Qian Y, Dana MR. Molecular mechanisms of immunity in corneal allotransplantation and xenotransplantation. *Expert Rev Mol Med* 2001; **2001**: 1-21.

117. Chitilian HV, Laufer TM, Stenger K, Shea S, Auchincloss H, Jr. The strength of cell-mediated xenograft rejection in the mouse is due to the CD4+ indirect response. *Xenotransplantation* 1998; **5**: 93-8.

118. Higuchi R, Streilein JW. CD8+ T cell-mediated delayed rejection of orthotopic guinea pig cornea grafts in mice deficient in CD4+ T cells. *Invest Ophthalmol Vis Sci* 2003; **44**: 175-82.

119. Tanaka K, Yamagami S, Streilein JW. Evidence that T-helper type 2 cell-derived cytokines and eosinophils contribute to acute rejection of orthotopic corneal xenografts in mice. *Transplantation* 2005; **79**: 1317-23.

120. Pindjakova J, Vitova A, Krulova M, Zajicova A, Filipec M, Holan V. Corneal rat-to-mouse xenotransplantation and the effects of anti-CD4 or anti-CD8 treatment on cytokine and nitric oxide production. *Transpl Int* 2005; **18**: 854-62.

Chapter 3

Immunomodulatory approaches

Various immunomodulatory approaches are applied in attempts to control allograft rejection with the aim to induce transplantation tolerance. Some of the approaches are presented here and those which involve corneal transplantation are set out in a more detailed manner.

CLINICAL PRACTICE

In clinical practice, topical prophylactic therapy with corticosteroids (prednisolone acetate or dexamethasone) is a standard postoperative treatment in normal- and high-risk patients and is used as treatment of established rejection episodes. Their application typically starts on the day of transplantation. In high-risk situations, in addition to glucocorticoids, immunosuppressants such as cyclosporine A (CsA), mycophenolate mofetyl (MMF) or methotrexat are the drugs of choice while the others are used only infrequently (tacrolimus or rapamycin).

Glucocorticoids

Corticosteroids exert a diversity of immunomodulatory actions as transcriptional regulators by binding to glucocorticoid response elements in the DNA or complexing with other regulatory elements. This allows simultaneous inhibition of several transcription factors (as nuclear factor kappa B (NFκB), activator protein-1 (AP-1) or signal transducers and activators of transcription (STATs)) and of downstream

inflammatory genes (Table 2) and provides broad immunosuppression - especially cellular as reviewed by Barnes (*1*)).

Table 2: The effect of corticosteroids on gene transcription (from Barnes (*1*))

Increased transcription

Lipocortin-1

b2-Adrenoceptor

Secretory leukocyte inhibitory protein

Clara cell protein (CC10)

IL-1 receptor antagonist

IL-1R2 (decoy receptor)

IjB-a

MIF

Decreased transcription

Cytokines

(IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-11, IL-12,

IL-13, TNF-a, GM-CSF, stem cell factor)

Chemokines

(IL-8, RANTES, MIP-1a, MCP-1, MCP-3, MCP-4, eotaxin)

iNOS

COX-2

Cytoplasmic PLA2

Endothelin-1

NK1-receptors, NK2-receptors

Adhesion molecules (ICAM-1, E-selectin)

While prophylactic treatment is mainly topical, treatment of acute endothelial rejection is often combined with other routes of application: per oral, intravenous (pulse therapy) or subconjunctival. This allows various levels of a systemic immunosuppression (*2*). Pulse therapy provides a significant advantage when initiated early after the onset of

rejection as prevention of subsequent rejection episodes (*3*). However, this therapy produces a variety of well-known side effects (both local and systemic) and other therapies have to be considered to enable the tapering of steroid's dose.

CsA

CsA is the second most preferred drug and is available for both local and systemic administration. It is the first T cell-selective drug which binds to cyclophilin and prevents the generation of nuclear factor of activated T cells (NF-AT), a factor crucial in the up-regulation of the mRNA of various cytokines - most importantly of IL-2 (necessary for the proliferation and maturation of T cells), and IFN-γ (critical for the activation of macrophages). Its main limits are especially in its nephrotoxicity, neurotoxicity and glucose intolerance and in its poor inhibition of humoral immunity (*4*). Addition of 2% CsA to standard topical corticosteroid treatment prolongs the rejection-free period but not the overall rejection rate (*5*). Topical 0.5% CsA alone was not proven better than corticosteroids (increased rejection rate) but it can be considered in the case of patients with secondary glaucoma. Results of systemic application after corneal transplantation provide conflicting results, but positive results predominate (*6, 7*).

MMF

MMF is usually the third drug of choice in high-risk corneal transplantation. After transformation in livers its active metabolite, mycophenolic acid, non-competitively inhibits the activity of inosine monophosphate dehydrogenase (IMPDH) during DNA synthesis. Due to a less active salvage pathway of the purine synthesis and increased demands during T and B cell activation, MMF acts as an antiproliferative drug. In high-

risk keratoplasty MMF proved equally effective in preventing acute rejection as systemic CsA treatment (*7*). In murine model of corneal transplantation the combination of MMF and rapamycin highly significantly prolonged graft survival (100 days versus 25.5 and 19.5 for rapamycin and MMF respectively) (*8*).

Tacrolimus (FK 506)

Tacrolimus acts similarly to CsA by forming a complex with FK-binding proteins (FK-BP) that inhibits phospatase activity of calcineurin and generation of NF-AT. Tacrolimus, better than CsA prolongs human liver graft survival but increases the risk of post-transplant diabetes (*9*). The first results in human corneal transplantation suggest that in high-risk grafts its systemic as well as local application is effective in reducing the rejection rate (*10, 11*). In mouse, combined therapy of tacrolimus and MMF did not show a synergistic effect (*12*); however its combination with anti-LFA-1 mAb highly significantly prolonged concordant xenograft survival (*13*).

Rapamycin (Rapamune, Sirolimus, RAD) and its analogues

Rapamycin is a macrolide immunosuppressant originally developed as anti-tumor (*14*) and anti-fungal (especially anti-candidal) agent (*15*). Although rapamycin, similarly to tacrolimus, binds to FK-BP 12, the resulting complex binds to mammalian target of rapamycin (mTor), blocks its activity and inhibits signals required for cell cycle progression, cell growth, and proliferation. In addition, it inhibits various cytokine-mediated signal transduction pathways, such as signaling from growth factor receptors (for example from IL-2R) (*16*). Its analogues like RAD (everolimus), CCI-779 or AP23573 are highly selective inhibitors of mTor (*17*). As mentioned above, in murine model of corneal transplantation RAD prolongs graft survival in monotherapy and

especially when used in combination with MMF. Similarly, in rat, combination with CsA had a synergic effect (*18*). RAD plays an important role in calcineurin inhibitor-sparing regimens (*19*).

Antiproliferative agents, cyclophosphamide and azathyoprine, produce potentially serious side effects (especially bone marrow suppression) and their usage is now restricted to cases refractory to other treatments or they are used in combination with other immunosuppressive drugs.

THERAPEUTIC APPROACHES – SCHEMATIC OVERVIEW OF EXPERIMENTAL RESULTS IN CORNEAL TRANSPLANTATION

(Only agents that are not used in clinical practice are presented in some detail.)

- **Antiproliferative agents** affecting lymphocyte cell division (such as azathioprine, cyclophosphamide, MMF or sirolimus).

- **Agents interrupting lymphocyte maturation** by:
    - **Alteration of cytokine synthesis** – calcineurin inhibitors (CsA, tacrolimus).
        - Occupation of surface receptors
            - Anti-CD25 mAb binds with high affinity with IL-2 receptor but does not activate it. Such inhibition limits clonal expansion of activated T cells and generation of CTL.
        - basiliximab (75% human, 25% murine)
        - daclizumab (90% human, 10% murine)

- Inhibition of signals required for cell cycle progression, cell growth and proliferation signal transduction phases of cytokine stimulation (sirolimus).
- Inhibition of cell differentiation (15-deoxyspergualin - a NF-kappa B inhibitor suppressing proinflammatory cytokines production and maturation of DC).
- Adoptive transfer of Tregs that in addition to production of suppressive mediators (mainly IL-10) may occupy/modulate available APC and compete with naïve cells for stimulatory signals (*20*).

- **Agents interrupting costimulation signals** between APC and lymphocytes. To achieve full activation, T lymphocytes have to receive several distinct coordinated "survival" signals. The first signal is provided following TCR receptor recognition of peptide/MHC complexes on APC. The second is delivered through the T cell costimulatory molecules (CD28 and CD40) by the interaction with their ligands (CD80 and 86 and CD40L) on various APC (DC, B cells, macrophages). In the absence of survival signals or their inappropriate timing T cells become unresponsive or anergic or deleted (see section CD154). This may lead to tolerance. Upon T cells activation, cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) is upregulated and has the ability to bind to CD80 and 86 molecules with 10 – 20 higher affinity than CD28. In contrast to CD28, CTLA-4 delivers a strong inhibitory signal that inhibits IL-2 and IL-2R expression and arrests T cells in G1 phase (*21*).
  - CD28 - B7 (CD80 and CD86) pathway
    - Anti-B7 (CD80 and CD86) and anti-CD28

- Systemic administration of anti-CD28 mAb to some extent prolongs survival of rat corneal allografts (*22*).

- CTLA-4
  - CTLA-4-Ig – a recombinant fusion protein composed of an immunoglobulin and CTLA-4
    - CTLA-4–Ig fusion protein was used in mouse, rat and rabbit models of corneal transplantation with various levels of effectivity. In mouse, *ex vivo* incubation of grafts for 24 hours with 10 μg/ml allowed survival beyond 100 days (*23*) while in rat, 2 or 10 μg/ml of CTLA-4-Ig for 18 hours prolonged survival for 6 and 2 days only (*24*). In rabbits 1, 10, 25, or 250 μg/ml of CTLA-4-Ig for 18 hours showed no differences when compared with control groups (*25*). In rats, however, corneal epithelium was removed before placing corneas into culture in order to limit the epithelium overgrowth. Such manipulation could possibly increase the risk of the graft rejection (*26*). In rabbits, the average number of rejected grafts in each group was two but the groups contained only 4 – 5 transplanted animals which make any conclusion regarding the efficacy difficult. More favorable results were achieved in the high-risk model where 1 and 10 μg/ml of CTLA-4-Ig resulted in about 50% of grafts surviving beyond 100 days in contrast to none in the control group (*25*). This positive effect was enhanced with additional UV-B graft irradiation before transplantation. Some prolongation of rat corneal allograft survival was also

achieved with single perioperative intraocular injection of CTLA-4-fusion protein with mutated (non-functional) immunoglobulin domains (*24*).

- Systemic administration of CTLA-4-Ig to some extent also prolonged survival of mouse and rat corneal allografts (*22, 27*).
- Combined treatment with CTLA-4-Ig and anti-CD154 mAb does not act synergistically in prolongation of corneal graft survival (*28*) in contrast to results obtained in other models (*29, 30*).

    - Betacept – has two amino acids substituted to increase its binding avidity (four times to CD86 and two times to CD80) with ten times greater inhibition of T cell activation and inhibition of anti-donor antibody formation (*31*).

- CD40 – CD40L (CD154) pathway
    - CD154 - is a T cell surface protein critical for the induction of CD4+ T cells response as well as for T cell mediated macrophage activation and T cell help of B cells. Experimental data of several authors support the concept that the blockage of costimulation by anti-CD154 mAb followed by anergy may actually lead to selective depletion of incompletely activated T cells rather than to their unresponsiveness (*32, 33*). Importantly, during early human clinical trials this treatment was associated with thrombo-embolic complications, which precluded their completion (CD154 is also

expressed on platelets). Concomitant administration of anti-inflammatory ketorolac may prevent this complication (*34*).

- Systemic (and to some extent also topical) anti-CD154 mAb treatment promoted murine corneal graft survival in both normal- and high-risk recipients (*35, 36*). The data also suggest that the early and prolonged administration is required (*35, 37*).
    - No synergic increase in survival was observed in the case of combined CTLA-4-Ig and anti-CD154 mAb treatment in murine corneal transplantation (*28*).

- **Agents interrupting cell adhesion**
    - Intercellular adhesion molecule–1 (ICAM-1)/LFA-1 interactions are critically involved in T cell activation and migration to target tissues in addition to their effect on B lymphocytes, natural killer cells, and granulocytes (*38, 39*).
        - ICAM-1
            - Anti-ICAM-1 antibody did not promote graft survival although it impaired CTL and DTH responses to donor alloantigens (*40*).
        - LFA-1
            - Anti-LFA-1, in contrast to anti-ICAM-1, prolongs murine corneal allograft survival (*41*) and prevents allosensitisation. However, it is not effective in previously immunized hosts (*40*).

- Anti-LFA-1 in combination with FK506 is highly effective in prolongation of corneal concordant xenografts survival (*13*).

- **Anticytokine agents**
  - Anti-TNF-α
    - Topical treatment with soluble tumor necrosis factor receptor type I prolonged corneal graft survival and inhibited gene expression of RANTES (CCL5) and MIF-1β associated with rejection (*42*).

  - Anti-IL-1αR
    - Topical treatment with anti-IL-1 receptor antagonist significantly promoted the acceptance of allogeneic corneal transplants in both normal- and high-risk transplantations and significantly reduced the levels of infiltrating Langerhans cells and suppressed allosensitisation (*43-45*).

  - IL-2-IgG2b fusion protein – subconjunctival or intraperitoneal administration to some extent prolonged corneal allograft survival (*46*).
  - cytotoxin IL-2-PE40 - a recombinant chimeric protein composed of IL-2 and a modified pseudomonas exotoxin
    - Intraperitoneal application significantly reduced the clinical rejection score and cumulative rejection rate in rat corneal allograft transplantation while reducing the total lymph node cell number and cytotoxic T cells activity in DLN (*47*).

- Anti-IL-15 - has IL-2-like functions but is not downregulated by CsA or tacrolimus

- **Agents depleting/modulating selected cell populations** bearing on its surface specific surface marker by lysis and/or inactivation (recently reviewed by Nahan (*48*)).
  - Anti-thymocyte globulin (equine ATGAM and rabbit Thymoglobulin).
  - Anti-CD3+ lymphocyte antibodies (OKT3, non-mitogenic hOKT3γ1 (Ala-Ala)).
  - Monoclonal anti-CD4+, CD8+ cells or pan-T cell antibody (F7D5, CAMPATH-1H).
    - Anti-CD52 mAb (CAMPATH-1H) was used successfully in one case of high-risk human corneal transplantation (*49*).

- **Agents affecting innate immune system**
  - Macrophages
    - iNOS inhibition
      - Within 4 weeks aminoguanidine (a specific iNOS inhibitor) prevented rejection in 57% versus 29% allografts in controls (*50*).
    - Subconjunctival injection of dichlormethylene diphosphonate (CL2MDP) encapsulated in liposomes prevented corneal allograft rejection (*51*) through depletion of macrophages. This treatment, however, affected already the afferent arm of the immune response by yet unexplained mechanism (*52*).

- **T_H2 modulation of the immune response**
    - Redirecting the recipient immune system toward the T_H2 response promoted acceptance of corneal allografts in high-risk settings (*53*).
    - Subconjunctival or intraperitoneal treatment with IL-10 did not prolong rat allograft survival and may have even accelerated it (*54*).

- **Inhibition of neovascularization**
    - Anti-vascular endothelial growth factor (VEGF) – suppressed vascular permeability and angiogenesis.
        - Anti-VEGF antibody topically applied significantly prolonged rat corneal allograft survival (*55*).

- **Induction of donor-specific ACAID**
    - Survival of mouse corneal allografts was increased by intracameral implantation of corneal allogeneic tissue when performed two (but not four) weeks after their implantation (*56*).
    - Intravenous administration of allogeneic peritoneal exudates cells incubated overnight with TGF-β followed after one week with mouse corneal transplantation in high-risk recipients (prevascularized) reduced the rejection rate (*57*).

- **Alteration of the immune cells trafficking**
    - FTY720 (Fingolimod)

- FTY720 is a sphingosine analog and an immunosuppressive drug with a novel mechanism of action. It acts on sphingosine 1-phosphate receptors (S1P-R). S1P-Rs are members of the endothelial differentiation gene (EDG) family of G protein–coupled receptors. They are implicated in various activities, including cell growth, cytoskeletal changes, migration, expression of adhesion molecule, survival, proliferation, and morphogenesis (recently reviewed by Hla (*58*)). Currently, however, the extent of the ability of FTY720 to affect these processes or the cell types involved is not well documented. Both sphingosine kinases (SphK) SphK1 and SphK2 can phosphorylate FTY720 in vitro (*59-61*), though *in vivo* only one experiment was done showing that SphK1 was not necessary for induction of lymphopenia (*62*). FTY720 in its phosphorylated form functions as an agonist on type 1, 3, 4 and 5 S1P receptors (*63, 64*), while non-phosphorylated FTY720 acts as a non-competitive inhibitor of type 1, 5 (with high potency) and 2 (with moderate potency) sphingosine receptors at nanomolar concentrations.

Although the mechanisms whereby FTY720 causes lymphopenia are not fully explained, several reports suggest that downregulation of $S1P_1$ receptors and their functional inactivation by FTY720 may contribute (*65-67*). It inhibits lymphocyte emigration from the peripheral lymphoid organs and the thymus (*68-72*), dramatically reducing the number of lymphocytes, especially T cells in the circulation (*68, 72, 73*), grafts (*73, 74*) and tissues (*75-77*). Sequestration of lymphocytes in lymph nodes and Peyer's patches (*78*) (although less pronounced in mouse (*79*)) is believed to be the dominant mode of action of FTY720

although long term treatment also reduces the proliferative response of mouse lymph node derived T cells to alloantigens (*79*).

Treatment with FTY720 has a beneficial effect in several other models of organ transplantation (*80-84*) including corneal allograft rejection (*85*), in models of autoimmune disease (*75*) and also for example in preventing the development of pathology in GvHD (*86*).

In the recently published results of phase III clinical study in *de novo* renal transplantation the immunosuppressive effect of 2.5 mg of FTY720 was comparable with MMF (both with full dose CsA). Exposure to FTY720 was associated with reduced creatinine clearance and, in 2.2% patients, with macular edema etiology of which has not yet been explained but may be related to the nature of kidney transplant recipients. On the other hand, the incidence of cytomegalovirus infections was higher with MMF treatment (*87*). At present, the phase III (FREEDOMS Study) in patients with relapsing-remitting multiple sclerosis is in progress.

- **Gene therapy**
  - T_H2 modulation of the immune response
    - AdrIL-4 *ex vivo* corneal gene transfer in rat model did not promote graft survival (*88, 89*). In sheep model, IL-4 gene transfer induced corneal eosinophilia.
    - AdvIL-10 *ex vivo* gene transfer prolonged corneal graft survival in sheep (*89*) but not in rat (own results + (*90*)) where only systemic

administration was successful (*90*). The opposite results may be related to the size of experimental animal, total antigen dose and the extent of surgical trauma which vIL-10 should cope with.

- T_H1 inhibition
  - AdIL-12-p40 that acts as natural antagonist of IL-12 *in vivo* doubled graft survival in sheep model (*89*).

- Inhibition of costimulation (signal 2)
  - Local and systemic AdCTLA-4-Ig gene transfer, similarly to vIL-10 local therapy, was much less effective (only modest prolongation) in contrast to highly successful single intraperitoneal administration one day before transplantation ($1x10^{10}$ PFU/ml) (*91*).
  - AdCTLA-4-Ig ($1x10^{9}$ PFU/ml) to some extent prolonged allograft survival after systemic intravenous administration one day after transplantation but the effect was minimal after corneal *ex vivo* transduction (*24*).
  - Ballistic CTLA-4 + IL-4 gene transfer into the lower lid (and similarly to corneal epithelium (*92*)) prolonged corneal graft survival. Importantly, authors also demonstrated the common lymphatic drainage of the cornea, lower lid and subconjunctival space (*92*).

- **Inhibition of CD4+ T cells proliferation**

- AdCD4GFP *ex vivo* gene transfer (cDNA encoding a monomeric anti-CD4 antibody fragment) did not affect graft survival (*93*). This anti-CD4 scFv was able to block *in vitro* lymphocyte activation in MLR. Postponing transplantation to three days after *ex vivo* transduction (in order to increase the amount of antibody produced at the time of antigen presentation) did not improve the outcome. Since adenoviral vectors transduce only endothelium, it seems unrealistic to expect that the sufficient amount of anti-CD4 antibody would get to DLN via the anterior chamber and blood circulation. Similarly, the production of anti-CD4 scFv may not be sufficiently high to inhibit CD4+ cells during the effector phase of the immune response because of the relatively short expression of the transgene.

- **Apoptosis induction**
  - AdTRAIL (Tumor necrosis factor-related apoptosis-inducing ligand) *ex vivo* transduction delayed rejection in murine model (*94*).

The development of new immunomodulatory strategies is directed toward approaches which produce less side effects and feature higher efficacy. The ultimate goal is the induction of transplantation tolerance. It is important to realize and take into consideration that the effects observed in rodent models are often not fully reproducible in humans. There are certain strategies in non-human primates (reviewed by Kean *et al.* (*95*)) that produce tolerance in a subset of recipients such as T cell costimulation blockage (anti-CD40L mAb + donor blood transfusion + rapamycin (*96*)),

induction of transient mixed chimerism (*97, 98*), T cell depletion (anti-CD3 immunotoxin + 15-deoxyspergualin) (*99, 100*) or the transfer of anergic T cells (*101*). However, in humans it may be necessary to combine various approaches. In addition, some of them may need to target the contribution of the innate immune system to the rejection.

REFERENCES

1. BARNES P. J. Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clin Sci (Lond)*, 1998, vol. 94, p. 557-572.
2. BERTELMANN E., REINHARD T., PLEYER U. [Current practice of immune prophylaxis and therapy in perforating keratoplasty. A survey of members of the Cornea Section of the German Ophthalmological Society]. *Ophthalmologe*, 2003, vol. 100, p. 1031-1035.
3. HILL J. C., MASKE R., WATSON P. Corticosteroids in corneal graft rejection. Oral versus single pulse therapy. *Ophthalmology*, 1991, vol. 98, p. 329-333.
4. LIU J. et al. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell*, 1991, vol. 66, p. 807-815.
5. INOUE K. et al. Long-term effects of topical cyclosporine A treatment after penetrating keratoplasty. *Jpn J Ophthalmol*, 2000, vol. 44, p. 302-305.
6. SUNDMACHER R., REINHARD T., HEERING P. Six years' experience with systemic cyclosporin A prophylaxis in high-risk perforating keratoplasty patients. A retrospective study. *Ger J Ophthalmol*, 1992, vol. 1, p. 432-436.
7. HILL J. C. Systemic cyclosporine in high-risk keratoplasty: long-term results. *Eye*, 1995, vol. 9 (Pt 4), p. 422-428.
8. REIS A. et al. Coadministration of the new macrolide immunosuppressant RAD and mycophenolate mofetil in experimental corneal transplantation. *Transplantation*, 2000, vol. 70, p. 1397-1401.
9. HADDAD E. M. et al. Cyclosporin versus tacrolimus for liver transplanted patients. *Cochrane Database Syst Rev*, 2006, vol., p. CD005161.
10. WANG M. et al. Studies on the effects of the immunosuppressant FK-506 on the high-risk corneal graft rejection. *Yan Ke Xue Bao*, 2002, vol. 18, p. 160-164.

11. SLOPER C. M., POWELL R. J., DUA H. S. Tacrolimus (FK506) in the management of high-risk corneal and limbal grafts. *Ophthalmology*, 2001, vol. 108, p. 1838-1844.

12. SVOZILKOVA P. et al. Comparison of FK 506, mycophenolate mofetil, and aminoguanidine effects on delay of corneal allograft rejection in an experimental model of low-risk and high-risk keratoplasty. *Immunopharmacol Immunotoxicol*, 2006, vol. 28, p. 335-340.

13. YAMAGAMI S. et al. Mechanism of concordant corneal xenograft rejection in mice: synergistic effects of anti-leukocyte function-associated antigen-1 monoclonal antibody and FK506. *Transplantation*, 1997, vol. 64, p. 42-48.

14. ENG C. P., SEHGAL S. N., VEZINA C. Activity of rapamycin (AY-22,989) against transplanted tumors. *J Antibiot (Tokyo)*, 1984, vol. 37, p. 1231-1237.

15. SEHGAL S. N., BAKER H., VEZINA C. Rapamycin (AY-22,989), a new antifungal antibiotic. II. Fermentation, isolation and characterization. *J Antibiot (Tokyo)*, 1975, vol. 28, p. 727-732.

16. HALLORAN P. F. Molecular mechanisms of new immunosuppressants. *Clin Transplant*, 1996, vol. 10, p. 118-123.

17. MITA M. M., MITA A., ROWINSKY E. K. Mammalian target of rapamycin: a new molecular target for breast cancer. *Clin Breast Cancer*, 2003, vol. 4, p. 126-137.

18. REIS A. et al. Synergism of RAD and cyclosporin A in prevention of acute rat corneal allograft rejection. *Cornea*, 2002, vol. 21, p. 81-84.

19. PASCUAL J. Everolimus in clinical practice--renal transplantation. *Nephrol Dial Transplant*, 2006, vol. 21 Suppl 3, p. iii18-23.

20. WALDMANN H. et al. Infectious tolerance and the long-term acceptance of transplanted tissue. *Immunol Rev*, 2006, vol. 212, p. 301-313.

21. KRUMMEL M. F., ALLISON J. P. CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. *J Exp Med*, 1996, vol. 183, p. 2533-2540.

22. THIEL M. A. et al. Local or short-term systemic costimulatory molecule blockade prolongs rat corneal allograft survival. *Clin Experiment Ophthalmol*, 2005, vol. 33, p. 176-180.

23. SHI W. Y., XIE L. X. [CTLA4-Ig prevents corneal allograft rejection in mice]. *Zhonghua Yan Ke Za Zhi*, 2004, vol. 40, p. 696-700.

24. COMER R. M. et al. Effect of Administration of CTLA4-Ig as Protein or cDNA on Corneal Allograft Survival. *Invest. Ophthalmol. Vis. Sci.*, 2002, vol. 43, p. 1095-1103.

25. GEBHARDT B. M. et al. Protection of corneal allografts by CTLA4-Ig. *Cornea*, 1999, vol. 18, p. 314-320.

26. HORI J., STREILEIN J. W. Role of recipient epithelium inpromoting survival of orthotopic corneal allografts in mice. *Invest. Ophthalmol. Vis. Sci.*, 2001, vol. 42, p. 720-726.

27. HOFFMANN F. et al. Inhibition of corneal allograft reaction by CTLA4-Ig. *Graefes Arch Clin Exp Ophthalmol*, 1997, vol. 235, p. 535-540.

28. ARDJOMAND N. et al. Modulation of Costimulation by CD28 and CD154 Alters the Kinetics and Cellular Characteristics of Corneal Allograft Rejection. *Invest. Ophthalmol. Vis. Sci.*, 2003, vol. 44, p. 3899-3905.

29. LARSEN C. P. et al. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature*, 1996, vol. 381, p. 434-438.

30. ELWOOD E. T. et al. Prolonged acceptance of concordant and discordant xenografts with combined CD40 and CD28 pathway blockade. *Transplantation*, 1998, vol. 65, p. 1422-1428.

31. LARSEN C. P. et al. Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. *Am J Transplant*, 2005, vol. 5, p. 443-453.

32. MONK N. J. et al. Fc-dependent depletion of activated T cells occurs through CD40L-specific antibody rather than costimulation blockade. *Nat Med*, 2003, vol. 9, p. 1275-1280.

33. HARGREAVES R. E., MONK N. J., JURCEVIC S. Selective depletion of activated T cells: the CD40L-specific antibody experience. *Trends Mol Med*, 2004, vol. 10, p. 130-135.

34. KOYAMA I. et al. Thrombophilia associated with anti-CD154 monoclonal antibody treatment and its prophylaxis in nonhuman primates. *Transplantation*, 2004, vol. 77, p. 460-462.

35. QIAN Y. et al. Blockade of CD40-CD154 Costimulatory Pathway Promotes Survival of Allogeneic Corneal Transplants. *Invest. Ophthalmol. Vis. Sci.*, 2001, vol. 42, p. 987-994.

36. QIAN Y., DANA M. R. Effect of locally administered anti-CD154 (CD40 ligand) monoclonal antibody on survival of allogeneic corneal transplants. *Cornea*, 2002, vol. 21, p. 592-597.

37. LARSEN C. P. et al. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. 1996, vol. 381, p. 434-438.

38. MARLIN S. et al. LFA-1 immunodeficiency disease. Definition of the genetic defect and chromosomal mapping of alpha and beta subunits of the lymphocyte function-associated antigen 1 (LFA-1) by complementation in hybrid cells. *J. Exp. Med.*, 1986, vol. 164, p. 855-867.

39. ANDERSON M. E., SIAHAAN T. J. Targeting ICAM-1/LFA-1 interaction for controlling autoimmune diseases: designing peptide and small molecule inhibitors. *Peptides*, 2003, vol. 24, p. 487-501.

40. HE Y. et al. Effect of LFA-1 and ICAM-1 antibody treatment on murine corneal allograft survival. *Invest Ophthalmol Vis Sci*, 1994, vol. 35, p. 3218-3225.

41. YAMAGAMI S. et al. The role of cell adhesion molecules in allograft rejection after penetrating keratoplasty in mice. Clinical and immunohistochemical study. *Graefes Arch Clin Exp Ophthalmol*, 1996, vol. 234, p. 382-387.

42. QIAN Y. et al. Topical soluble tumor necrosis factor receptor type I suppresses ocular chemokine gene expression and rejection of allogeneic corneal transplants. *Arch Ophthalmol*, 2000, vol. 118, p. 1666-1671.

43. YAMADA J. et al. Interleukin-1 receptor antagonist therapy and induction of anterior chamber-associated immune deviation-type tolerance after corneal transplantation. *Invest Ophthalmol Vis Sci*, 2000, vol. 41, p. 4203-4208.

44. YAMADA J. et al. Interleukin 1 receptor antagonist suppresses allosensitization in corneal transplantation. *Arch Ophthalmol*, 1998, vol. 116, p. 1351-1357.

45. DANA M. R., YAMADA J., STREILEIN J. W. Topical interleukin 1 receptor antagonist promotes corneal transplant survival. *Transplantation*, 1997, vol. 63, p. 1501-1507.

46. ZHANG E. P. et al. Prolongation of corneal allograft survival by an interleukin-2-immunoglobulin fusion protein in mice. *Graefes Arch Clin Exp Ophthalmol*, 1998, vol. 236, p. 486-492.

47. HERBORT C. P. et al. Treatment of corneal allograft rejection with the cytotoxin IL-2-PE40. *Transplantation*, 1991, vol. 52, p. 470-474.

48. NASHAN B. Antibody induction therapy in renal transplant patients receiving calcineurin-inhibitor immunosuppressive regimens: a comparative review. *BioDrugs*, 2005, vol. 19, p. 39-46.

49. NEWMAN D. K. et al. Prevention of immune-mediated corneal graft destruction with the anti-lymphocyte monoclonal antibody, CAMPATH-1H. *Eye*, 1995, vol. 9 (Pt 5), p. 564-569.

50. STRESTIKOVA P. et al. FK 506 and aminoguanidine suppress iNOS induction in orthotopic corneal allografts and prolong graft survival in mice. *Nitric Oxide*, 2003, vol. 9, p. 111-117.

51. VAN DER VEEN G. et al. Prevention of corneal allograft rejection in rats treated with subconjunctival injections of liposomes containing dichloromethylene diphosphonate. *Invest Ophthalmol Vis Sci*, 1994, vol. 35, p. 3505-3515.

52. SLEGERS T. P. et al. Macrophages play a role in the early phase of corneal allograft rejection in rats. *Transplantation*, 2004, vol. 77, p. 1641-1646.

53. YAMADA J. et al. Mice with Th2-Biased Immune Systems Accept Orthotopic Corneal Allografts Placed in "High Risk" Eyes. *J Immunol*, 1999, vol. 162, p. 5247-5255.

54. TORRES P. F. et al. Interleukin 10 treatment does not prolong experimental corneal allograft survival. *Ophthalmic Res*, 1999, vol. 31, p. 297-303.

55. YATOH S. et al. Effect of a topically applied neutralizing antibody against vascular endothelial growth factor on corneal allograft rejection of rat. *Transplantation*, 1998, vol. 66, p. 1519-1524.

56. SONODA A. et al. ACAID induced by allogeneic corneal tissue promotes subsequent survival of orthotopic corneal grafts. *Invest Ophthalmol Vis Sci*, 2000, vol. 41, p. 790-798.

57. SANO Y., OKAMOTO S., STREILEIN J. W. Induction of donor-specific ACAID can prolong orthotopic corneal allograft survival in "high-risk" eyes. *Curr Eye Res*, 1997, vol. 16, p. 1171-1174.

58. HLA T. Physiological and pathological actions of sphingosine 1-phosphate. *Semin Cell Dev Biol*, 2004, vol. 15, p. 513-520.

59. PAUGH S. W. et al. The immunosuppressant FTY720 is phosphorylated by sphingosine kinase type 2. *FEBS Lett*, 2003, vol. 554, p. 189-193.

60. BILLICH A. et al. Phosphorylation of the immunomodulatory drug FTY720 by sphingosine kinases. *J Biol Chem*, 2003, vol. 278, p. 47408-47415.

61. SANCHEZ T. et al. Phosphorylation and action of the immunomodulator FTY720 inhibits vascular endothelial cell growth factor-induced vascular permeability. *J Biol Chem*, 2003, vol. 278, p. 47281-47290.

62. ALLENDE M. L. et al. Mice deficient in sphingosine kinase 1 are rendered lymphopenic by FTY720. *J Biol Chem*, 2004, vol.

63. BRINKMANN V. et al. The immune modulator FTY720 targets sphingosine 1-phosphate receptors. *J Biol Chem*, 2002, vol. 277, p. 21453-21457.

64. MANDALA S. et al. Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science*, 2002, vol. 296, p. 346-349.

65. MATLOUBIAN M. et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature*, 2004, vol. 427, p. 355-360.

66. ALLENDE M. L. et al. Expression of the sphingosine 1-phosphate receptor, S1P1, on T-cells controls thymic emigration. *J Biol Chem*, 2004, vol. 279, p. 15396-15401.

67. GRALER M. H., GOETZL E. J. The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G protein-coupled receptors. *FASEB J.*, 2004, vol., p. 03-0910fje.

68. MIYATA Y. et al. Prolongation of concordant xenograft survival by treatment with FTY720. *Transplant Proc*, 1998, vol. 30, p. 4163-4165.

69. MAEDA A. et al. Immunosuppression with FTY720 and cyclosporine A inhibits rejection of adult porcine islet xenografts in rats. *Transplantation*, 2003, vol. 75, p. 1409-1414.

70. FUJIWARA I. et al. Prolongation of concordant xenograft survival by a newly developed drug, FTY720. *Transplant Proc*, 1999, vol. 31, p. 2831-2833.

71. FUKUDA Y. et al. Effect of FTY720 on immunoregulation in concordant xenotransplantation. *Transpl Int*, 1998, vol. 11 Suppl 1, p. S461-464.

72. YAGI H. et al. Immunosuppressant FTY720 inhibits thymocyte emigration. *Eur J Immunol*, 2000, vol. 30, p. 1435-1444.

73. YANAGAWA Y. et al. FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. II. FTY720 prolongs skin allograft survival by decreasing T cell infiltration into grafts but not cytokine production in vivo. *J Immunol*, 1998, vol. 160, p. 5493-5499.

74. UEDA H. et al. Effect of a novel immunosuppressant, FTY720, on allograft survival after renal transplant in rats. *Eur Surg Res*, 2000, vol. 32, p. 279-283.

75. SUZUKI K. et al. Prevention of experimentally induced autoimmune type I diabetes in rats by the new immunosuppressive reagent FTY720. *Transplant Proc*, 1998, vol. 30, p. 1044-1045.

76. HOZUMI Y. et al. Immunotherapy for experimental rat autoimmune thyroiditis using a novel immunosuppressant, FTY720. *Life Sci*, 1999, vol. 65, p. 1739-1745.

77. MIYAMOTO T. et al. Therapeutic effects of FTY720, a new immunosuppressive agent, in a murine model of acute viral myocarditis. *J Am Coll Cardiol*, 2001, vol. 37, p. 1713-1718.

78. CHIBA K. et al. FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. I. FTY720 selectively decreases the number of circulating mature lymphocytes by acceleration of lymphocyte homing. *J Immunol*, 1998, vol. 160, p. 5037-5044.

79. LUO Z. J. et al. Analysis of the mode of action of a novel immunosuppressant FTY720 in mice. *Immunopharmacology*, 1999, vol. 41, p. 199-207.

80. SUZUKI S. et al. Immunosuppressive effect of a new drug, FTY720, on lymphocyte responses in vitro and cardiac allograft survival in rats. *Transpl Immunol*, 1996, vol. 4, p. 252-255.

81. SUZUKI S. et al. An immunosuppressive regimen using FTY720 combined with cyclosporin in canine kidney transplantation. *Transpl Int*, 1998, vol. 11, p. 95-101.

82. SUZUKI S. et al. A novel immunosuppressant, FTY720, with a unique mechanism of action, induces long-term graft acceptance in rat and dog allotransplantation. *Transplantation*, 1996, vol. 61, p. 200-205.

83. MASUBUCHI Y. et al. FTY720, a novel immunosuppressant, possessing unique mechanisms. IV. Prevention of graft versus host reactions in rats. *Transplant Proc*, 1996, vol. 28, p. 1064-1065.

84. XU M. et al. Effect of peritransplant FTY720 alone or in combination with posttransplant FK 506 in a rat model of cardiac allotransplantation. *Transplant Proc*, 1997, vol. 29, p. 2964-2966.

85. ZHANG E. P. et al. Significant prolongation of orthotopic corneal-graft survival in FTY720-treated mice. *Transplantation*, 2003, vol. 76, p. 1511-1513.

86. MITSUSADA M. et al. Prevention of graft rejection and graft-versus-host reaction by a novel immunosuppressant, FTY720, in rat small bowel transplantation. *Transpl Int*, 1997, vol. 10, p. 343-349.

87. MULGAONKAR S. et al. FTY720/cyclosporine regimens in de novo renal transplantation: a 1-year dose-finding study. *Am J Transplant*, 2006, vol. 6, p. 1848-1857.

88. PLEYER U. et al. Survival of corneal allografts following adenovirus-mediated gene transfer of interleukin-4. *Graefes Arch Clin Exp Ophthalmol*, 2000, vol. 238, p. 531-536.

89. KLEBE S. et al. Prolongation of sheep corneal allograft survival by ex vivo transfer of the gene encoding interleukin-10. *Transplantation*, 2001, vol. 71, p. 1214-1220.

90. GONG N. et al. Effects of local and systemic viral interleukin-10 gene transfer on corneal allograft survival. *Gene Ther*, 2006, vol.

91. GONG N. et al. Influence of local and systemic CTLA4Ig gene transfer on corneal allograft survival. *J Gene Med*, 2006, vol. 8, p. 459-467.

92. ZHANG E. P. et al. Ballistic CTLA4 and IL-4 gene transfer into the lower lid prolongs orthotopic corneal graft survival in mice. *Graefes Arch Clin Exp Ophthalmol*, 2003, vol. 241, p. 921-926.

93. JESSUP C. F. et al. Local gene transfer to modulate rat corneal allograft rejection. *Invest. Ophthalmol. Vis. Sci.*, 2005, vol. 46, p. 1675-1681.

94. GUO P. et al. [The effect of TNF-related apoptosis-inducing ligand on in vivo graft survival after ex vivo adenovirus-mediated gene transfer to cornea]. *Zhonghua Yan Ke Za Zhi*, 2003, vol. 39, p. 19-23.

95. KEAN L. S. et al. Transplant tolerance in non-human primates: progress, current challenges and unmet needs. *Am J Transplant*, 2006, vol. 6, p. 884-893.

96. PRESTON E. H. et al. IDEC-131 (anti-CD154), sirolimus and donor-specific transfusion facilitate operational tolerance in non-human primates. *Am J Transplant*, 2005, vol. 5, p. 1032-1041.

97. KAWAI T. et al. Costimulatory blockade for induction of mixed chimerism and renal allograft tolerance in nonhuman primates. *Transplant Proc*, 2001, vol. 33, p. 221-222.

98. KIMIKAWA M. et al. Mixed chimerism and transplantation tolerance induced by a nonlethal preparative regimen in cynomolgus monkeys. *Transplant Proc*, 1997, vol. 29, p. 1218.

99. THOMAS J. M. et al. Peritransplant tolerance induction in macaques: early events reflecting the unique synergy between immunotoxin and deoxyspergualin. *Transplantation*, 1999, vol. 68, p. 1660-1673.

100. THOMAS J. M. et al. Successful reversal of streptozotocin-induced diabetes with stable allogeneic islet function in a preclinical model of type 1 diabetes. *Diabetes*, 2001, vol. 50, p. 1227-1236.

101. BASHUDA H. et al. Renal allograft rejection is prevented by adoptive transfer of anergic T cells in nonhuman primates. *J Clin Invest*, 2005, vol. 115, p. 1896-1902.

Experimental work

Chapter 4

The establishment of the corneal transplantation model in mouse and rat

4.1.1 Allotransplantation model

(Mouse to mouse; BALB/c to C57BL/6 and rat to rat; Wistar Furth to Lewis)

4.1.2 Concordant xenotransplantation model

(Rat to mouse; Lewis to BALB/c and Sprague Dawley to BALB/c)

Corneal transplantation was performed in accordance with the report by She *et al.* (44), with some modifications. Briefly, a 2 or 3.5 mm trephine was used to mark the mouse or rat donor cornea respectively and a sharp needle was used to enter the anterior chamber. To complete the dissection micro scissors were used and the graft was stored in balanced salt solution at room temperature until transplantation. Recipients were anesthetized with a mixture of ketamin and xylazin intraperitonealy. Tropicamidum and phenylephrine hydrochloride were used to perform the mydriasis (brand names differ according to the manuscripts). The corneal graft was fixed in place by interrupted or running (in rat) 11/0 Ethilon black monofilament non-absorbable suture (Johnson & Johnson Intl., UK). At the end of the procedure, an antibiotic ointment was applied. Sodium Hyaluronate (10 mg/ml) was used in the course of

surgery in order to protect the corneal endothelium. The sutures were left in place for the duration of the experiment.

Technical improvement was achieved by the introduction of a needle with small diameter (50 μm in contrast to previously available 150 μm) which enables easier suturing and creates smaller perforation channels. This reduces leakage and iris anterior synechiae formation typically associated with increased risk of rejection (*1*)). However, there is a disadvantage in that the point of the needle is not as sharp as that of the Sharpoint 150 μm needle and, accordingly, stronger force is required for piercing the cornea. In general, we prefer the use of nylon sutures which are soft and shapeable in contrast to rigid Mersilen suture endings of which point sharply against palpebral conjunctiva and irritate it. This is clinically associated with pronounced palpebral erythema and edema. When interrupted sutures are used, sutures endings can be left longer which enables their later removal (around day 7-8), or they can be trimmed as short as possible and then they remain in place. The advantage of the latter technique is in the early reduction of irritation induced by long stitches. Results of our experiment exploring the influence of the suturing technique in xenotransplantation model (for more details see Chapter 5) suggest that suture length by itself may have an important impact on graft survival. In mouse model, we prefer using interrupted sutures. Here, according to our opinion, a running suture does not allow precise adaptation of the wound, has a tendency towards loosening (at least in parts of the graft) and because the whole suture is secured only by one stitch, suture endings cannot be left too short (due to the risk of untying). This invariably leads to irritation and induction of neovascularization. The situation is better in rat, where the diameter of the graft is bigger (∅ 3.5 mm) but even here later induction of neovascularization towards the graft can occur.

4.2 Grading schemes for clinical course after corneal grafting

In the model of corneal transplantation, all types of typical corneal graft rejection can be detected (endothelial, stromal and epithelial). Except for endothelial or epithelial rejection, histological detection is necessary. Since the introduction of rat or mouse corneal transplantation model, several grading schemes were suggested (for example (2-4)). In general, they focus on grading of corneal opacity (as a marker of endothelial cells dysfunction) and sometimes of graft edema, infiltration and neovascularization (its extent and activity). They differ in the number of grades covering the full extent of the possible clinical picture and/or in which structure they use for comparison. Importantly, these details are not crucial for comparison between different studies.

In mouse model, we eventually had to choose the combination BALB/c (donors) and C57BL/6 (graft recipients) (disparate in both MHC class I and II) in order to get reasonable percentage of rejecting grafts instead of the opposite combination often preferred by other groups. Consequently, we had to adapt the scheme formerly used in our laboratory (2) for recipients with dark iris.

After some time, it became obvious that the evaluation of the iris vessels reaction can be a good indicator of forthcoming rejection. Therefore, for the purposes of grading we divided the clinical picture into four grades. The scheme was used, for example, in experiments examining the influence of IL-4 gene transfer on corneal allograft survival (Chapter 9).

Note: If necessary, fraction of the whole digit was used to describe the clinical picture precisely (i.e. opacity 0.5).

Motivated by the need to compare our results with results of other groups, we respected the grading schemes already published by Hašková *et al.* (*2*) and did not alter them unless it was beneficiary.

4.2.1 Corneal opacity

Corneal opacity resulting from corneal edema and/or cellular infiltration is usually graded as "total opacity" because it is difficult to distinguish which component is responsible. Corneal transparency is mainly dependent on the function of endothelial pump. Situations adversely affecting endothelium (such as surgical trauma, rejection, high intraocular pressure) are associated with swelling of the corneal stroma or, when prominent, with bullous keratopathy. We thought initially that cellular infiltration looks like grayishness but the results of our IL-4 *ex vivo* gene transfer experiment may suggest that it looks rather like corneal granularity. Some authors base their grading of corneal opacity solely on the intensity of stromal opacity (for example (*4*)) but we prefer using either iris vessels or pupil details (in dark pigmented mice). Our approach seems preferable because results obtained by the first grading method may be affected by the angle of observation, light conditions or the anterior chamber reaction forming the background for observation.

- BALB/c recipients

The opacity of grafts were assessed according this grading score: grade 0, clear graft; grade 1, minor opacity with the structure of the anterior chamber is clearly distinguishable; grade 2, opaque stroma with the vessels of iris and pupil visible; grade 3, opaque stroma with at least part of the pupil visible and grade 4, opaque stroma, with undistinguishable structures in the anterior chamber.

Opacity above grade 1 was considered as the onset of rejection for the purpose of the manuscript presented in *Chapter 6* (*5*). Alternatively, opacity higher than 0 can be used, as well. The "day of rejection" was usually set as the day when the opacity of the cornea increased to the extent that only pupil margin remained distinguishable (opacity > 2.5) unless specified differently.

- *C57BL/6 recipients*

C57BL/6 mice have pigmented iris which precludes the evaluation of iris vessels details (which are an indirect sign of corneal opacity). Therefore, we had to modify the previous grading scheme of corneal opacity:

Grade 0, clear graft; grade 1, minor opacity with the pupil margin clearly distinguishable; grade 2, opaque stroma with the pupil margin visible; grade 3, opaque stroma with at least part of the pupil visible and grade 4, opaque stroma, with undistinguishable structures in the anterior chamber.

The "day of rejection" is set as the day when the opacity of the cornea increased to the extent that only the pupil margin remained distinguishable (opacity > 2.5).

4.2.2 Corneal neovascularization

In order to assess the **extent of corneal neovascularization**, we used a grading scheme ranging from 0 to 2. Grade 0 is assigned when no neovascularization is present, grade 0.5 and 1 when new vessels reached half the distance between the limbus and the graft margin or the graft margin respectively. Additionally, grading can

be extended to grade 1.5 and 2 when less (1.5) or more (2) than half of the transplant periphery is involved.

"The activity of neovascularization" is graded as follows: grade 0.4 for vessels with uneven "obliterated" lumen; grade 0.6 for narrow and 0.8 for dilated vessels and grade 1.0 for greatly dilated vessels.

4.2.3 Reaction of iris vessels

For assessment of the **iris vessels reaction** it was necessary to establish a new grading scheme. For this purpose, we modified the activity of corneal neovascularization scheme. Grade 0.4, caliber of the vessels smaller than in grade 0.5; grade 0.5 caliber of the vessels in normal healthy, non-transplanted rat; grade 0.8, highly dilated iris vessels; grades 0.6 and 0.7, caliber of the vessels equally distributed between grades 0.5 and 0.8.

4.3 The clinical course after corneal allograft transplantation

(Untreated transplanted animals; additional details are in *Chapter 7* and *9*)

- BALB/c to C57BL/6 (interrupted stitches with short endings)
- Wistar Furth to Lewis rat (running suture)

Day 1 to 5 – 6 – postoperative corneal edema and opacity plus the reaction in the anterior chamber (vasodilatation of iris vessels) (Figure 1A and B). A clear trend to a milder reaction is observed as surgical skills improve or when a rat cornea (which is more rigid) is used.

Day 3 to 4 – a usual time of the onset of corneal neovascularization (Figure 1C).

Day 7 to 11 – corneal neovascularization reaches the graft/host interface. Until then, it is uncommon to see an increase in corneal opacity (in contrast to presensitized hosts (*6*)). Such an extent of neovascularization does not always occur, it can reach a certain level (i.e. grade 0.5) after which it starts regressing. In such a case, 2 to 5 days before the onset of corneal opacification, you can see active vessels reaching the graft interface again, which later progress into the graft. At some point during the time between the day 7 to day 11, a small peak in corneal opacification in a previously clear cornea can be sometimes observed which, however, ceases after few days. This may represent a nonspecific reaction to a suture material or a surgical trauma because this reaction can be observed after syngeneic transplantation, as well.

Day 2 to 3 before the onset of rejection - apparent vasodilatation of iris vessels and often also a slowly progressive deepening of the anterior chamber. There is no report

on the level of the intraocular pressure in this situation in mouse or rat. In addition, vasodilatation of new corneal vessels suggests the level of the activity of the process.

"Day of rejection" – several criteria were used previously to best reflect the experimental design (rejection index or opacity score) (for example (*3, 4, 7*)). Usually, a corneal opacity degree 1, 2 or 3 is chosen. In our experiments, we preferred using a degree 3 (i.e. when at least a part of the pupil is visible) which makes a more consistent judgment of the score possible. As the time progresses, the opacity and corneal edema cease but the graft does not regain its original appearance. Usually, it rather looks like a wrinkled cellophane foil (this late phase is not covered in Figure 1). Similarly to the graft opacity decreasing through time, corneal neovascularization also diminishes. The grafts are usually examined at least three times a week and, when rejection is ongoing, daily. The timing can be modified in order to best suit the conditions of the experiment.

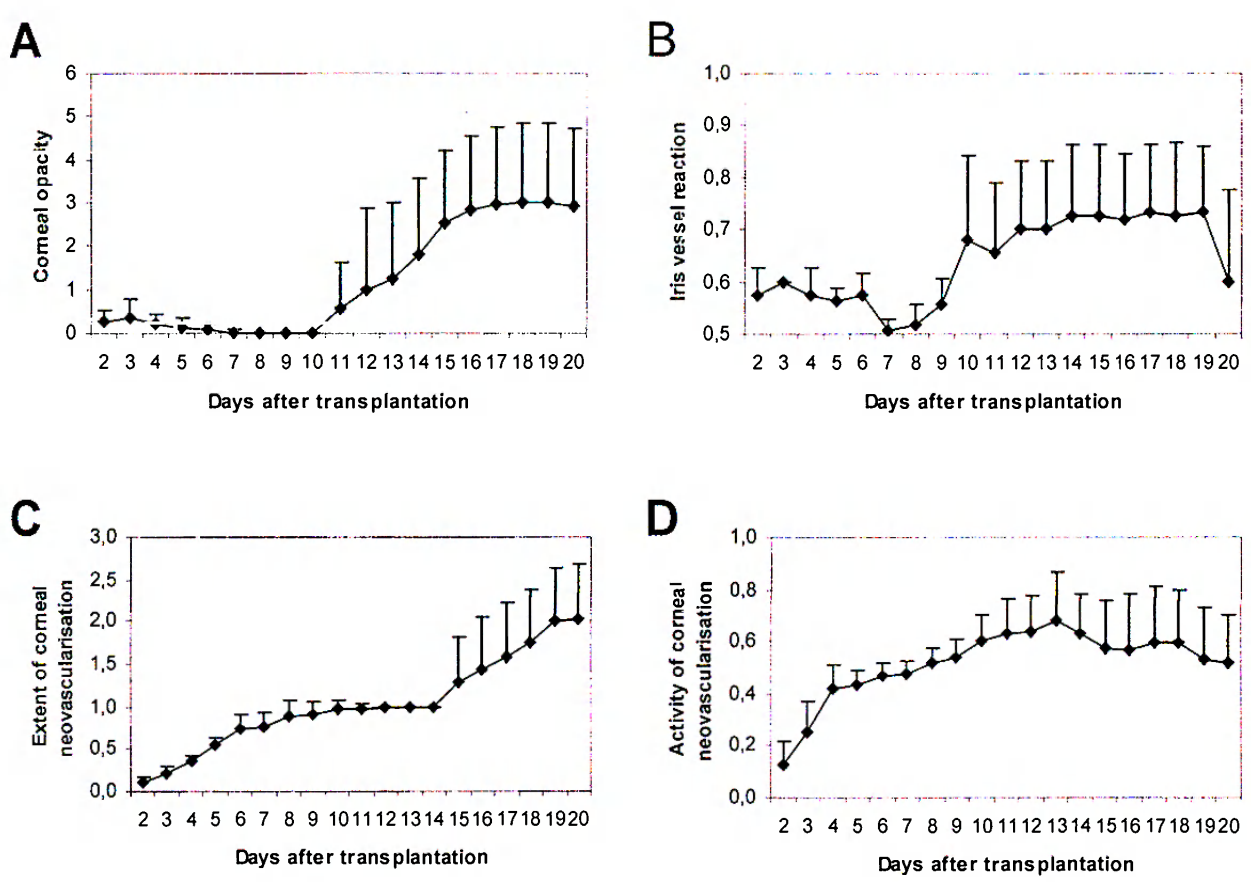


Figure 1: Examples of the kinetics of corneal opacity (A), the iris vessels reaction (B), the extent of corneal neovascularization (C) and the corneal neovascularization activity (D) after allotransplantation (Wistar Furth → Lewis rat).

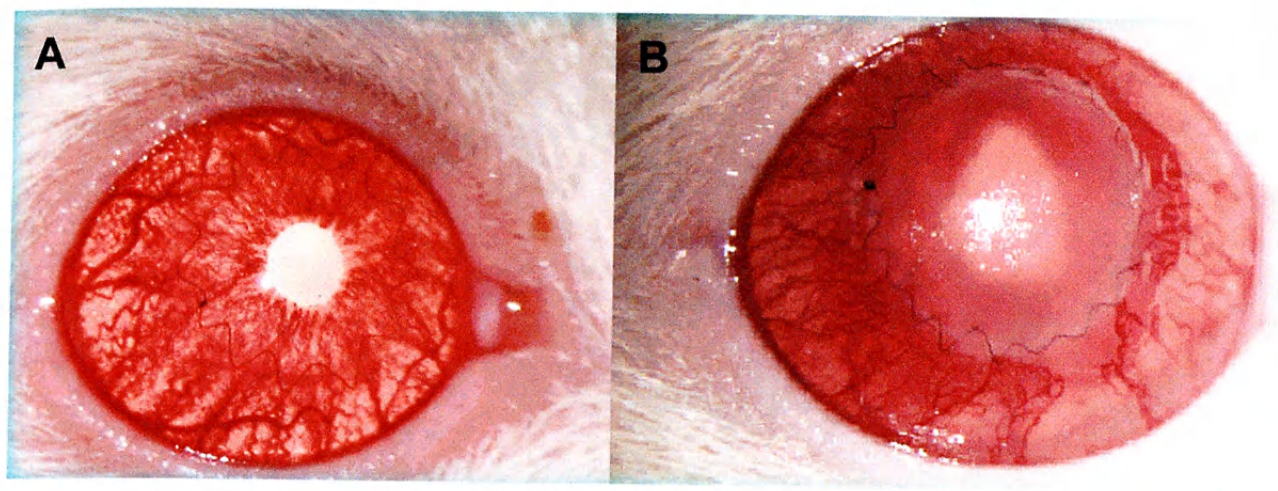


Figure 2: Examples of a clear (opacity 0; A) and of a rejected corneal graft (opacity 3; B) after allotransplantation (Wistar Furth → Lewis rat).

4.4 The clinical course after corneal concordant xenograft transplantation

(Untreated transplanted animals; additional details are in *Chapter 5* and *6*)

- Lewis → BALB/c (interrupted stitches with long or short endings) – *Chapter 5*
- Sprague Dawley → BALB/c (interrupted stitches with short endings) – *Chapter 6*

Day 1 to 3 - 5 – minimal postoperative corneal edema and opacity (Figure 3A) and a minimal reaction in the anterior chamber (vasodilatation of iris vessels). Better surgical skills significantly limit the extent of the postoperative inflammatory reaction.

Day 2 to 3 – a usual time of the onset of corneal neovascularization (Figure 3B).

Day 6 to 10 – corneal neovascularization reaches the area of the graft/host interface (this occurs earlier when long suture endings are used (for details see *Chapter 5*)). Neovascularization of the graft is observed very rarely unless immunosuppression is used (for example *Chapter 6*). In this model, it is not advisable to remove the corneal sutures because this often leads to the dehiscence of the wound straight afterwards or during the following days because of the very intensive rejection reaction at the graft interface.

"Day of rejection" – a similar description to that stated in the above allotransplantation section, but the intensity of the overall inflammatory reaction is very prominent. This is especially marked at the graft/host interface where tissue disintegration is apparent. The usual tempo of the corneal opacity increase during

rejection is one degree per day (Figure 3A) in contrast to a slower pace in allotransplantation models. From day 3 or 4 after the transplantation, the grafts are usually examined daily under the operating microscope. With the passage of time, the tissue of the graft seems to be "shrinking" (Figure 4B) and the surrounding tissue becomes restored almost to its original condition within 2 to 3 months, with some brownish intrastromal deposits (Figure 4C). This is a preliminary result which shows the substantial reparative capability the mouse tissue features and which warrants further research.

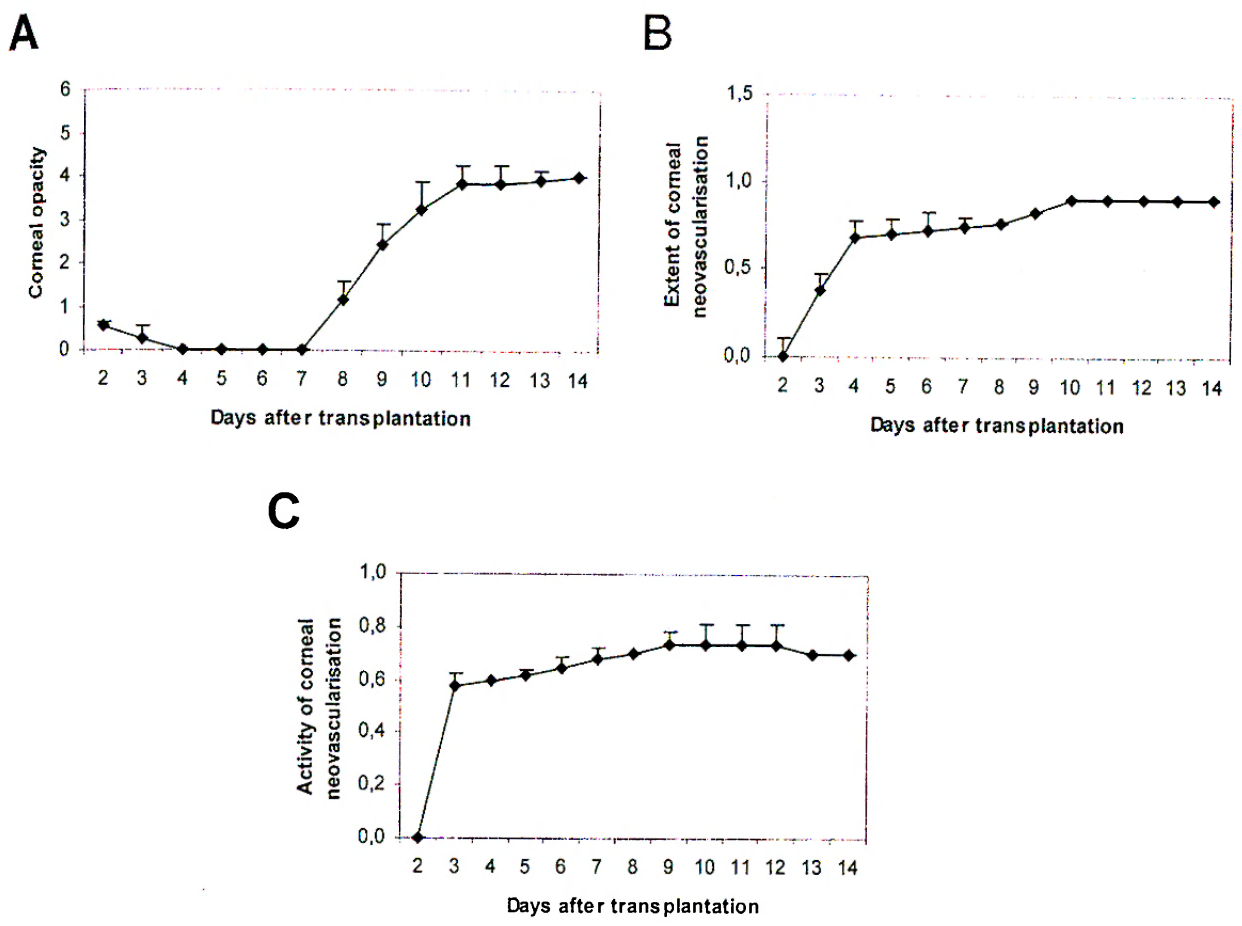


Figure 3: Examples of the corneal opacity kinetics (A), the iris vessels reaction (B), and the extent of corneal neovascularization (C)) after concordant xenotransplantation (Lewis → BALB/c, short suture endings).

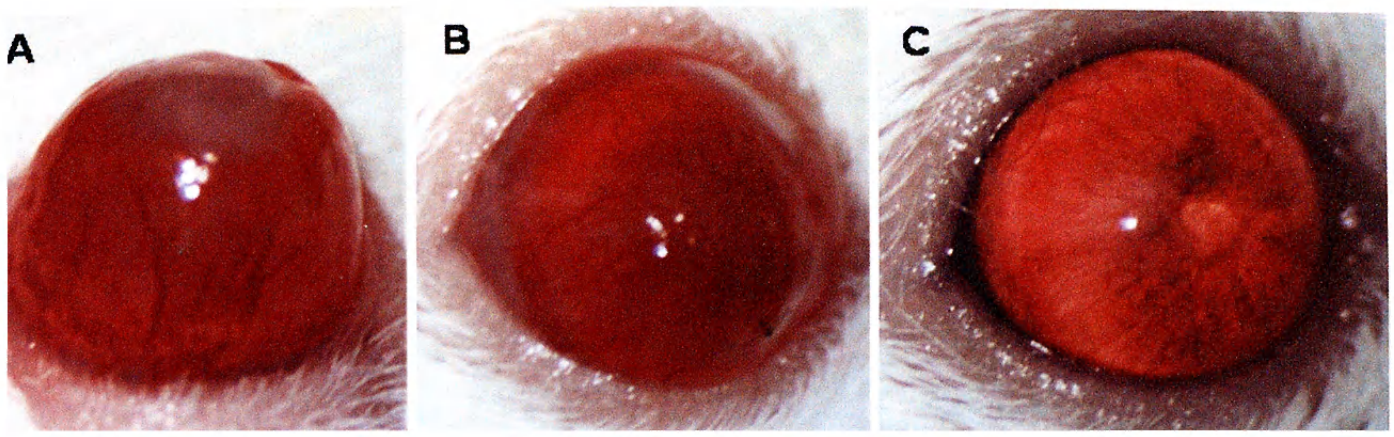


Figure 4: The clinical appearance of xenograft rejection (A), one (B) and two months (C) after transplantation. (Lewis → BALB/c, short suture endings).

REFERENCES

1. YAMAGAMI S., TSURU T. Increase in orthotopic murine corneal transplantation rejection rate with anterior synechiae. *Invest Ophthalmol Vis Sci*, 1999, vol. 40, p. 2422-2426.
2. HASKOVA Z., FILIPEC M., HOLAN V. [The significance of gender incompatibility in donors and recipients and the role of minor histocompatibility antigens in corneal transplantation]. *Ces Slov Oftalmol*, 1997, vol. 53, p. 128-135.
3. HE Y. G., ROSS J., NIEDERKORN J. Y. Promotion of murine orthotopic corneal allograft survival by systemic administration of anti-CD4 monoclonal antibody. *Invest Ophthalmol Vis Sci*, 1991, vol. 32, p. 2723-2728.
4. SANO Y., KSANDER B. R., STREILEIN J. W. Fate of orthotopic corneal allografts in eyes that cannot support anterior chamber-associated immune deviation induction. *Invest Ophthalmol Vis Sci*, 1995, vol. 36, p. 2176-2185.
5. SEDLAKOVA K. et al. FTY720 in corneal concordant xenotransplantation. *Transplantation*, 2005, vol. 79, p. 297-303.
6. NICHOLLS S. M., BRADLEY B. B., EASTY D. L. Effect of mismatches for major histocompatibility complex and minor antigens on corneal graft rejection. *Invest Ophthalmol Vis Sci*, 1991, vol. 32, p. 2729-2734.
7. HASKOVA Z., FILIPEC M., HOLAN V. The role of major and minor histocompatibility antigens in orthotopic corneal transplantation in mice. *Folia Biol (Praha)*, 1996, vol. 42, p. 105-111.

Chapter 5

The Effect of the Suturing Technique on Corneal Xenograft Survival

Klara Sedlakova and Martin Filipec

Submitted for publication

The Effect of the Suturing Technique on Corneal Xenograft Survival

Klara Sedlakova, M.D.[a] and Martin Filipec, M.D.[b,c]

[a] Department of Ophthalmology, 1st Medical Faculty, Charles University and General Teaching Hospital, Prague, Czech Republic

[b] Laboratory of the Biology and Pathology of the Eye, Institute of Inherited Metabolic Disorders, 1st Medical Faculty, Charles University and General Teaching Hospital, Prague, Czech Republic

[c] Eye Clinic Lexum, Prague, Czech Republic

Corresponding author: M. Filipec, Eye Clinic Lexum, U Společenské zahrady 3, Prague 4 - Krč, Czech Republic. Phone: (+420) 244 016 441, Fax: (+420) 244 016 440; email: murfi@centrum.cz.

Key Words: Corneal xenotransplantation - Surgical technique - Immunosuppression - Graft survival

ABSTRACT

Purpose. Understanding xenograft rejection is crucial for the potential introduction of xenotransplantation into clinical practice. Small animal models play an essential role in this context and significantly contribute to our knowledge about mechanisms of xenograft rejection. **Methods.** Rat to mouse corneal xenografts were performed using two suturing techniques. Sutures were left either long or as short as possible to limit the extent of a non-specific inflammatory response. Cyclosporine A (CsA), monoclonal antibody anti-T cells and a specific inhibitor of inducible nitric oxide synthase (alone or in a combination with CsA) were tested as immunosuppressants. **Results.** Grafts with long sutures were rejected in 7.3 ± 1.2 days, whereas those with short sutures after 11.8 ± 1.0 days ($p < 0.001$). Similarly, long sutures induced more pronounced corneal neovascularization ($p < 0.001$). While in groups of recipients with long sutures all tested immunosuppressants significantly ($p < 0.01 – 0.001$) prolonged corneal graft survival, none of them showed a comparable efficacy in groups of recipients with short sutures. **Conclusion.** This study demonstrates that the suturing technique significantly affects the outcome of corneal concordant xenograft transplantation, influences the effectiveness of immunosuppressive regimens and therefore must be taken into account when evaluating their efficacy.

Corneal transplantation represents the most frequent type of transplantation of solid tissue. In many countries, there becomes a shortage of donor material. Xenotransplantation is regarded as a possible future solution. Potential feasibility of genetic manipulation seems to further support this assumption. However, many factors, such as genetic disparity between a donor and a recipient, immunosuppression used or a surgical technique, can influence the outcome of xenotransplantation. Porcine corneas are considered the most probable future source of xenografts in humans, but performed anti-pig antibodies may possibly threaten the outcome of the transplantation. The majority of human anti-pig natural antibodies are directed against one particular carbohydrate determinant - α-galactoside. However, there is experimental evidence that porcine corneas lack α-gal epitope (except for several keratocytes in the most anterior part)[1,2] and therefore xenoantibodies should not probably represent a prominent problem in human corneal transplantation. For this reason, we selected concordant model.

The surgical technique plays an important role; in particular with the decreasing size of animal models. While in humans or rabbits are used corneal grafts 8 mm in diameter, in rats or in mice it is 3 or 2 mm. Even now, we do not know whether and to what extent the suturing technique can affect the outcome of corneal xenotransplantation. The innate immune system facilitates dendritic cell trafficking to lymph nodes, leukocyte migration into the graft and, during the later phase, it acts synergistically with the acquired immunity to reject the xenograft. When considering the usage of interrupted sutures, two techniques may be employed. Sutures can be left longer which allows their later removal, or they can be trimmed as short as possible and then remain in place. Both suturing techniques are used standardly in mice due to the small corneal thickness that precludes

knot's burring. Clinically, the advantage of the latter technique is in the reduction of the irritation induced by long stitches.

Xenograft rejection is a complex reaction involving both the innate and acquired immune responses and is accompanied by a pronounced inflammatory response in the grafted eye. Large numbers of cells are present in the anterior chamber and significant numbers of lymphocytes and cells of the monocyte/macrophage lineage invade the surrounding tissue and, to a lesser extent, the graft.[3,4] Although mechanisms of xenograft rejection differ from allograft rejection,[5,6] similarities do exist. Hyperacute or acute vascular rejections, described in the case of other solid organ xenotransplants, do not occur in this model. The rejection reaction is mediated primarily by CD4+ T cells with a minor contribution of complement but other innate and/or adaptive immune effectors must play role.[7]

Immunosuppressive monotherapy has not been reported to be particularly effective in delaying corneal xenograft rejection. One promising agent seems to be FTY720 which substantially suppresses the severity of the inflammatory response and dose dependently postpones rejection.[4] Since corneal graft rejection involves mainly T cells and macrophages producing nitric oxide (NO),[8-10] we tested effects of calcineurin inhibitor cyclosporine A (CsA), monoclonal antibody (mAb) anti-Thy-1.2 and a specific inhibitor of inducible NO synthase (iNOS) on corneal xenograft rejection in two groups of recipients. The recipients differ in the type of the suturing technique.

The results showed that the suturing technique significantly affects the outcome of corneal xenograft transplantation and must be taken into consideration when evaluating the efficacy of immunosuppressive therapy.

MATERIALS AND METHODS

Animals

The inbred strain of BALB/c mice (8-12 weeks old) was used as recipients of Lewis rat corneal grafts. Mice were obtained from the breeding unit of the Institute of Molecular Genetics, Academy of Science, Prague and rats were purchased from the Institute of Physiology, Academy of Science, Prague. Syngeneic transplantations were performed on BALB/c mice. All animals were treated in accordance with the regulations of the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research.

Corneal transplantation

Orthotopic corneal transplantation was performed according to our standard protocol with some modifications. Briefly, recipients were anesthetized with a mixture of Narkamon 5% (ketaminum 5%, Spofa, Praha, Czech Republic) and Rometar 2% (xylazin, Spofa, Praha, Czech Republic) diluted in saline intramuscularly. Mydrum (tropicamidum, Ankerpharm, Rudolstadt, Germany) and Neo-Synephrine (phenylephrine hydrochloride, Sanofi, Chicago, IL) were used to induce mydriasis. The corneal graft (∅ 2 mm) was fixed in place by interrupted 11/0 Ethilon black monofilament non-absorbable sutures (Johnson & Johnson Intl., Yorkshire, UK) that were left in place for the duration of the experiment. Two suturing techniques were used: sutures were left either long (Fig. 1A) or as short as possible (Fig. 1B) in order to decrease the extent of non-specific inflammatory response. At the end of transplantation an antibiotic ointment [Ophthalmo-Framykoin (polymyxin B sulphate and bacitricin zinc), Zentiva, Prague, Czech Republic] was applied. Provisc (sodium hyaluronate 10 mg/ml, Alcon, Puurs, Belgium) was used in the course of surgery to protect the corneal endothelium. The

"day of the rejection" was set as the day when the opacity of the cornea increased to the extent that only pupil margin remained distinguishable (opacity > 2.5). In order to evaluate the extent of corneal neovascularization on day 8 after transplantation, we used a grading scheme ranging from 0 to 2. Grade 0 was assigned when no neovascularization was present, grade 0.5 and 1 when new vessels reached half the distance between the limbus and the graft margin or the graft margin respectively and grades 1.5 and 2 when less (1.5) or more (2) than half of the transplant periphery was involved.

Drug administration and treatment protocol

A specific inhibitor of iNOS, 2-amino-5.6-dihydro-6-methyl-4-H-1.3-thiazine (AMT) (Sigma Chemical Company, St. Luis, MO),[11] was administered at a dose of 5 mg/kg/day [n = 8 (long sutures) and 9 (short sutures)]. Monoclonal antibody (mAb) against anti-Thy 1.2 antigen (clone F7D5)[12] (100 μg/mouse/day) (n = 8 and 5) and AMT were both administered intraperitonealy once a day. Cyclosporine (CsA) (Sandimmune Neoral oral solution, Novartis, Basel, Switzerland) (25 mg/kg/day), a kind gift of Novartis, Czech Republic, was administered daily orally (n = 6 and 11). CsA was given alone or in combination with AMT (n = 7 and 10 respectively). Untreated animals which received the xenogeneic graft (n = 16 and 9) were used as controls. All treatments began on the day of transplantation.

Statistical analysis

The Kaplan-Meier test was used to determine the statistical significance of the suturing technique on the kinetics of corneal grafts rejection and the Mann Whitney test to ascertain the extent of corneal neovascularization. The Kruskal-Wallis test was used to determine the statistical significance of the suturing technique on the effectiveness of different immunosuppressive compounds in terms of the delay of corneal graft rejection. Probability values below 0.05 were considered statistically significant.

RESULTS

Effect of the suturing technique on corneal xenograft survival

To ascertain the effect of different suturing techniques on corneal graft survival, we compared two groups of mice: one where corneal grafts were sutured with long stitches (Fig. 1A) and one where the grafts were sutured with short stitches (Fig. 1B). Mice with sutures only partially trimmed rejected their grafts between 6 to 9 days after transplantation, i.e. in 7.4 ± 1.2 (7.5) days [mean survival time ± SD (median value)]. However, when sutures were trimmed as much as possible, rejection occurred about 5 days later, in 11.8 ± 1.0 (12) days. This difference in graft surviving times (Fig. 2A) was statistically significant ($p < 0.001$). In addition, long sutures induced more pronounced corneal neovascularization – mean neovascularization grade 1.9 ± 0.2 versus 1.0 ± 0.4 in recipients with short sutures ($p < 0.001$) when compared on day 8 (Fig. 2B).

The role of the suturing technique in assessing efficacy of immunosuppressive therapy

In order to compare the efficacy of selected immunosuppressants under different suturing conditions, two techniques of interrupted sutures were used as described above and four immunosuppressive regimens were compared to non-treated corneal xenografts: AMT (inhibitor of iNOS), F7D5 (mAb anti-T cells), CsA and CsA + AMT. In groups with long sutures all immunosuppressants significantly ($p < 0.01 – 0.001$) prolonged corneal graft survival (Fig. 3A). Furthermore, the combination of CsA + AMT significantly ($p < 0.001$) prolonged graft survival in comparison to results reached when each drug was used in monotherapy. Survival time for controls and AMT, F7D5, CsA and CsA + AMT treated groups were 9.8 ± 2.0 (9.5),

9.9 ± 1.0 (10), 10.1 ± 1.2 (10) and 13.0 ± 2.0 (12) days respectively. Interestingly, none of the immunosuppressive compounds significantly extended graft survival in groups with short sutures (Fig. 3B) where survival times for the above mentioned groups were 11.7 ± 1.6 (12), 13.4 ± 1:5 (13), 13.7 ± 4.7 (13) and 13.7 ± 3.3 (13) days.

DISCUSSION

Effective treatment of the rejection reaction requires our understanding of immune processes after transplantation and animal models play an important role in this regard. Small animal models are beneficial in many respects but, at the same time, they become technically more demanding as the size of the models decreases. The surgical technique thus becomes a critical factor influencing the outcome of transplantation. In this study we demonstrate that a simple alteration in the suturing technique can lead up to a 60% (4.5 days) difference in the mean corneal xenograft survival time and even more importantly that it affects the efficacy of immunosuppressive therapy.

In the present experiments, we used two interrupted suturing techniques in order to examine their effect on graft survival in a model of concordant xenograft transplantation. Our recent results show that using maximally trimmed suture endings and thus limiting the degree of non-specific irritation causes a significant delay in the onset of rejection as well as decreases the induction of neovascularization. These results are supported by clinical experience which demonstrates that loose sutures increase the risk of graft rejection and have potential to induce neovascularization and lymphangiogenesis.[13]

To explore further the impacts of the suturing technique, we wished to ascertain whether the efficacy of selected immunosuppressants follows the same pattern in both suturing techniques. We used a classical immunosuppressive drug CsA and mAb against T cells (anti-Thy-1.2). In addition, we aimed to find out whether treatment with AMT, a specific inhibitor of iNOS, alone or in combination with CsA, affects xenograft survival. The rationale to use AMT as immunosuppressant was based on the previous experiments demonstrating that inhibition of iNOS by a specific inhibitor delayed corneal allograft rejection in mice.[9]

The results showed that while in groups of corneal xenograft recipients with long sutures all tested immunosuppressants significantly ($p < 0.01 – 0.001$) prolonged graft survival, none of them showed similar efficacy in groups of recipients with short sutures. Altogether, the data showed that the treatment with CsA or mAb anti-Thy-1.2 had comparable immunosuppressive effects on corneal xenograft survival as did inhibition of iNOS. This observation is in an agreement with our previous results demonstrating that the activation of iNOS is a T cell-dependent process.[14] It suggests that NO is involved in corneal xenograft rejection, but it is not by far the only mechanism of rejection. Our data may therefore suggest that a substantial part of the immunosuppressive potency of selected immunosuppressants subsists in limiting the contribution of antigen-nonspecific inflammation and, consequently, that the dominant effector mechanisms responsible for xenograft rejection are not effectively compromised by this treatment. It is very likely that in order to achieve accommodation of xenografts, simultaneous blockage of several immune pathways will be necessary.

The results of this study demonstrate that the suturing technique significantly affects the outcome of corneal concordant xenograft transplantation. Importantly, it influences the effectiveness of immunosuppressive regimens and therefore must be taken into account when evaluating the efficacy of immunosuppressive drugs.

Acknowledgement: This work was supported by the grant NR/7816-3 from the Ministry of Health of the Czech Republic. The authors would like to thank Assoc. Prof. Vladimir Holáň (Institute of Molecular Genetics, Academy of Science of the Czech Republic, Prague, Czech Republic) for critical reading of the manuscript.

LEGENDS TO FIGURES

FIG. 1. Two different suturing techniques of corneal grafts used in this study. Corneal grafts were sutured with either stitches only partially trimmed (long stitches; A) or trimmed as much as possible (short stitches; B) (Illustrative photos).

FIG. 2. The influence of different suturing techniques (see legend to **FIG. 1**.) on survival of Lewis rat corneal grafts in BALB/c mice. The effects of long or short stitches on graft survival (**A**) and on extent of corneal neovascularization (**B**) are demonstrated. Each bar represents mean value + standard deviation (* $p < 0.001$).

FIG. 3. The effect of two different suturing techniques on the efficacy of selected immunosuppressive compounds. The graft recipients were either untreated or were treated with AMT (a specific inhibitor of iNOS), F7D5 (mAb anti-Thy 1.2), CsA (cyclosporine A) or a combination of CsA + AMT. In the groups of recipients with long sutures (**A**) all immunosuppressants significantly prolonged corneal xenograft survival, while no significant effect of the compounds on corneal graft survival was seen in the groups with short sutures (**B**). Small horizontal lines represent median value of the graft survival (* $p < 0.01$ and ** $p < 0.001$ vs. control; ° $p < 0.01$ and °° $p < 0.001$ vs. group indicated by connecting lines).

Figures

Figure 1

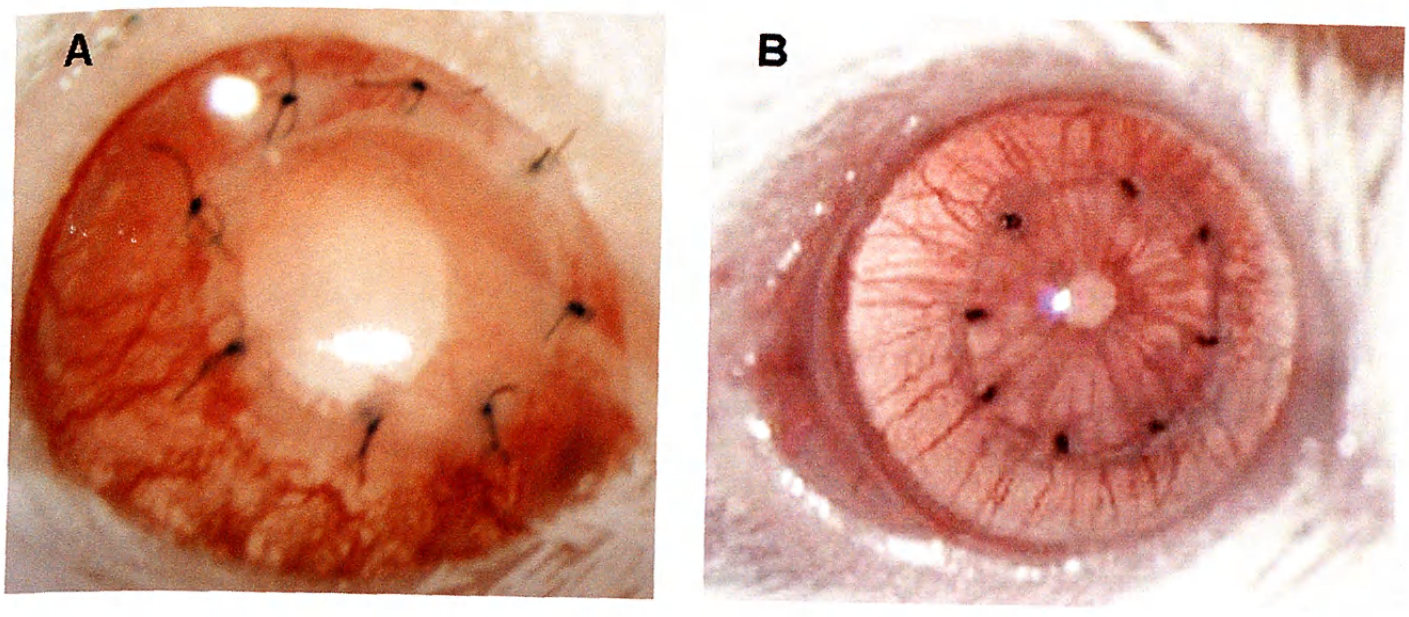


Figure 2

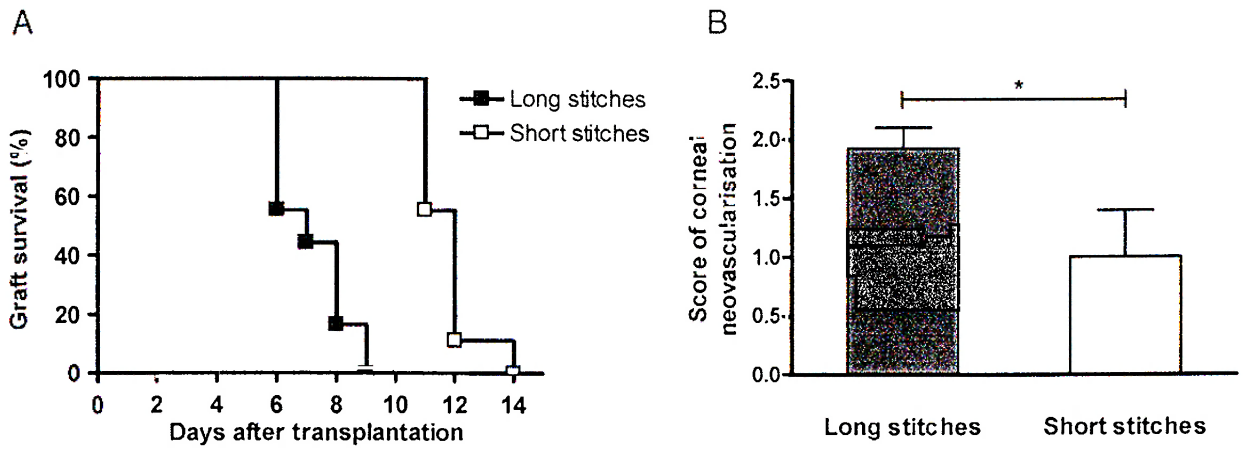
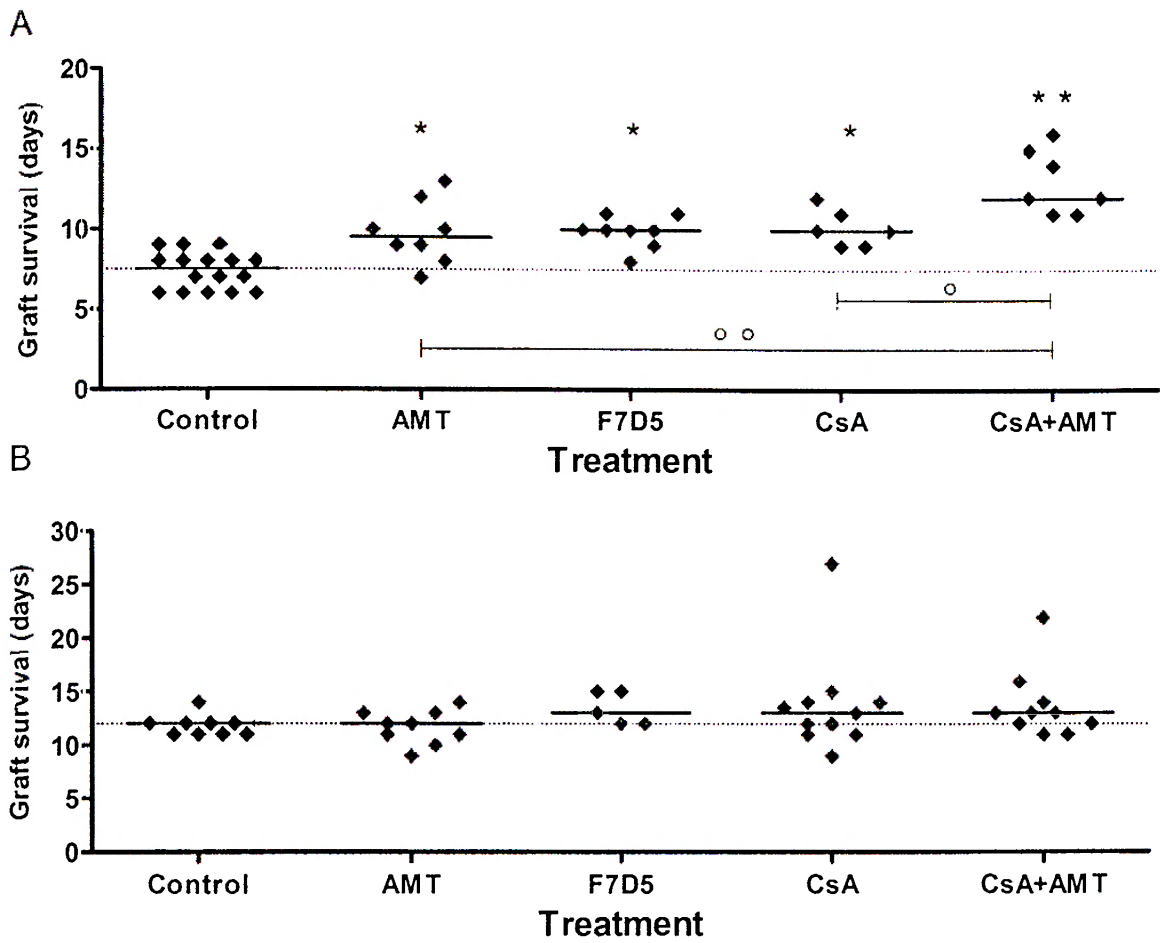

A
Graft survival (%)
100
80
60
40
20
0
0 2 4 6 8 10 12 14
Days after transplantation
Long stitches
Short stitches
B
Score of corneal neovascularisation
2.5
2.0
1.5
1.0
0.5
0.0
*
Long stitches
Short stitches

Figure 3


A
Graft survival (days)
20
15
10
5
0
Control
AMT
F7D5
CsA
CsA+AMT
Treatment
B
Graft survival (days)
30
25
20
15
10
5
0
Control
AMT
F7D5
CsA
CsA+AMT
Treatment

REFERENCES

1. Hrdlickova-Cela E, Smetana K, Jr., Plzak J, et al. Cells of porcine epidermis and corneal epithelium are not recognized by human natural anti-alpha-galactoside IgG. *Folia Biol (Praha)* 2001;47:200.

2. Amano S, Shimomura N, Kaji Y, et al. Antigenicity of porcine cornea as xenograft. *Curr Eye Res* 2003;26:313.

3. Yamagami S, Isobe M, Yamagami H, et al. Mechanism of concordant corneal xenograft rejection in mice: synergistic effects of anti-leukocyte function-associated antigen-1 monoclonal antibody and FK506. *Transplantation* 1997;64:42.

4. Sedlakova K, Muckersie E, Robertson M, et al. FTY720 in corneal concordant xenotransplantation. *Transplantation* 2005;79:297.

5. Qian YDana M. Molecular mechanisms of immunity in corneal allotransplantation and xenotransplantation. *Expert Reviews in Molecular Medicine* 2001;3:1.

6. Schuurman HJ, Cheng JLam T. Pathology of xenograft rejection: a commentary. *Xenotransplantation* 2003;10:293.

7. Tanaka K, Yamada JStreilein JW. Xenoreactive CD4+ T cells and acute rejection of orthotopic guinea pig corneas in mice. *Invest Ophthalmol Vis Sci* 2000;41:1827.

8. Kuffova L, Holan V, Lumsden L, et al. Cell subpopulations in failed human corneal grafts. *Br J Ophthalmol* 1999;83:1364.

9. Strestikova P, Plskova J, Filipec M, et al. FK 506 and aminoguanidine suppress iNOS induction in orthotopic corneal allografts and prolong graft survival in mice. *Nitric Oxide* 2003;9:111.

10. Pindjakova J, Vitova A, Krulova M, et al. Corneal rat-to-mouse xenotransplantation and the effects of anti-CD4 or anti-CD8 treatment on cytokine and nitric oxide production. *Transpl Int* 2005;18:854.

11. Nakane M, Klinghofer V, Kuk JE, et al. Novel potent and selective inhibitors of inducible nitric oxide synthase. *Mol Pharmacol* 1995;47:831.

12. Lake P, Clark EA, Khorshidi M, et al. Production and characterization of cytotoxic Thy-1 antibody-secreting hybrid cell lines. Detection of T cell subsets. *Eur J Immunol* 1979;9:875.

13. Cursiefen C, Maruyama K, Jackson DG, et al. Time course of angiogenesis and lymphangiogenesis after brief corneal inflammation. *Cornea* 2006;25:443.

14. Krulova M, Zajicova A, Fric J, et al. Alloantigen-induced, T-cell-dependent production of nitric oxide by macrophages infiltrating skin allografts in mice. *Transpl Int* 2002;15:108.

Chapter 6

FTY720 in Corneal Concordant Xenotransplantation

Klára Sedláková, Elizabeth Muckersie, Marie Robertson, Martin Filipec and John V. Forrester

(SEDLÁKOVA K. et al. FTY720 in corneal concordant xenotransplantation. Transplantation, 2005, vol. 79, p. 297-303.)

FTY720 in Corneal Concordant Xenotransplantation

Klára Sedláková [1, 2, 3], Elizabeth Muckersie[1], Marie Robertson[1], Martin Filipec[2, 3] and John V. Forrester [1]

[1] Department of Ophthalmology, University of Aberdeen, AB25 2ZD, UK

[2] 1. LF UK, Charles University, Kateřinská 32, Prague 2, 128 08, Czech Republic

[3] Department of Ophthalmology, VFN, U Nemocnice 2, Prague 2, 128 08, Czech Republic

Keywords: xenotransplantation – cornea – FTY720 - mouse - rat – epithelium

Word Counts:

Abstract – 250 words

Text – 3572 words

Tables: 1

Figures: 4

Correspondence should be addressed to: J.V. Forrester, Department of Ophthalmology, IMS, University of Aberdeen, Medical School, AB25 2ZD, j.forrester@abdn.ac.uk

Footnotes:

Work was supported by the Development Trust, University of Aberdeen.

Abbreviations: CSLN – cervical superficial lymph node, HD – high dose, LD – low dose, LN – lymph node, SMLN – submandibular lymph node

ABSTRACT (word count: 250)

Background. Currently there is no effective treatment for control of corneal xenograft rejection. We evaluated the efficacy and mode of action of a novel immunosuppressant, FTY720, in a model of corneal xenograft transplantation.

Methods. Rat to mouse corneal xenografts were performed and the effects of treatment with daily intraperitoneal injections of FTY720 (0.5 or 3.0 mg/kg/d) or saline from 2 days before transplantation were assessed clinically. Immunohistochemical studies of the grafts and flow cytometry of the draining lymph node subpopulations were performed at the time of clinical rejection.

Results. Treatment with FTY720 delayed the onset of corneal rejection from 8 days post-graft in saline treated mice to 12.0±0.89 (low dose) and 15.6±3.1 (high dose) days (both $p<0.001$). Histologically, FTY-treated animals had a markedly reduced inflammatory response in the anterior chamber and the cornea with replacement of the xenograft epithelium with normal healthy host epithelium. In contrast, saline treated xenografts had persisting corneal epithelial defects and ulceration. In the draining lymph nodes, FTY720 not only inhibited the increase in the cell number observed in saline treated recipients of xenografts, but also reduced the expression of activation markers on B cells (MHC class II and CD86).

Conclusions. FTY720 treatment significantly delayed rejection and decreased its severity in a dose dependent manner in a rat-to-mouse model of corneal xenotransplantation. Since corneal xenograft rejection is mediated not by natural antibodies or CD8+ T cells directly, but by CD4+ T cells, the data from these experiments imply that FTY720 mediated its effect via CD4+ T cells.

The one year outcome of human corneal transplantation is generally excellent (1), although non HLA-matched grafts are used predominantly. However, the four year survival rate is relatively poor, and may be less than the equivalent rate for some vascularized grafts (2, 3). In addition, there are several situations in which the prognosis for graft survival remains unsatisfactory including corneal neovascularization, active inflammation, or a previous history of corneal graft rejection, all considered to be "high risk" grafts (2, 4). Furthermore, despite the fact that corneal grafts are the most frequently transplanted tissue (1), there is a continuing shortage of donor material. Accordingly, corneal xenografts have long been considered a possibility as early as 1838 (R. Kissam).

The mechanisms responsible for xenograft rejection have been investigated in corneal transplants in experimental animals [reviewed in (*5*)]. The immunoprivileged environment of the cornea appears to provide corneal xenogeneic grafts with some degree of protection, as hyperacute or acute vascular rejection as described for other solid organ transplants does not occur here (*6*). The rejection of corneal transplants is a slower process and is mediated by CD4+ T cells with a minor contribution from complement (*7*). CD8 T cells and NK T cells are not obligatory, but may play a role in rejection in situations where CD4+T cells are absent or their function is impaired (*8, 9*). The role of the humoral immune response in corneal xenograft rejection is more controversial. Accumulation of antibodies in both allografts and xenografts has been shown (*6, 10, 11*) but rejection of xenografts in mice deficient in the μ heavy chain of immunoglobulin gene is not delayed (*7*). In contrast, the experiments in corneal allografts using B-cell knockout (*12, 13*) or deficient (*13*) mice showed that, although antibodies were not necessary for corneal rejection, their presence could produce an additional extensive injury in a complement-dependent manner.

Corneal xenograft rejection is generally resistant to treatment with single immunomodulatory agents such as FK506 (*6*), LFA-1 (*6*), cyclosporine (CsA), 2-amino-5, 6-dihydro-6-methyl-4H-1, 3-thiazine (AMT; an inhibitor of inducible NO synthase) or antibodies directed against Thy-1.2 (F7D5) (our unpublished results). In this study, we investigated the efficacy of FTY720, a sphingosine analog and an immunosuppressive drug with a novel mechanism of action, acting on sphingosine 1-phosphate receptors (S1P-R). S1P-Rs are members of the endothelial differentiation gene (EDG) family of G protein–coupled receptors. They are implicated in various activities, including cell growth, cytoskeletal changes, migration, expression of adhesion molecule, survival, proliferation, and morphogenesis (recently reviewed in reference (*14*)). Currently however, the extent of the ability of FTY720 to affect these processes or the cell types involved is not well documented. Both sphingosine kinases (SphK) SphK1 and SphK2 can phosphorylate FTY720 in vitro (*15-17*), though in vivo only one experiment was done showing that SphK1 was not necessary for induction of lymphopenia (*18*). FTY720 in its phosphorylated form functions as an agonist on type 1, 3, 4 and 5 S1P receptors (*19, 20*), while non-phosphorylated FTY720 acts as a non-competitive inhibitor of type 1, 5 (with high potency) and 2 (with moderate potency) sphingosine receptors at nanomolar concentrations.

Although the mechanisms whereby FTY720 causes lymphopenia in not fully explained, several reports suggest that down-regulation of $S1P_1$ receptors and their functional inactivation by FTY720 may contribute (*21-23*).

Treatment with FTY720 has a beneficial effect in several other models of organ transplantation (*24-28*) including corneal allograft rejection (*29*), in models of autoimmune disease (*30*) and also in preventing the development of pathology in GvHD (*31*). It inhibits lymphocyte emigration from the peripheral lymphoid organs

and the thymus (*32-36*), dramatically reducing the number of lymphocytes, especially T cells in the circulation (*32, 36, 37*), grafts (*37, 38*) and tissues (*30, 39, 40*). Sequestration of lymphocytes in lymph nodes and Peyer's patches (*41*) (although less pronounced in mice (*42*)) is believed to be the dominant mode of action of FTY720 although long term treatment also reduces the proliferative response of mouse lymph node derived T cells to alloantigens (*42*).

We report that although treatment with FTY720 alone does not influence graft survival in other xenogenic transplantation models (*32-35, 43*), it significantly delays the rejection of rat-to-mouse xenogenic concordant corneal transplants.

MATERIALS AND METHODS

Animals

BALB/c mice (10 to 18 weeks of age) were used as recipients and corneal grafts were prepared from Sprague-Dawley rats (4 weeks old). One eye of the recipient underwent corneal transplantation. Animals were obtained from the Biological Services Unit, University of Aberdeen or purchased from Harlan UK Ltd. and handled in accordance with the regulations of the United Kingdom Animal License Act 1986 and ARVO Statement for the Use of Animals in Ophthalmic and Visual Research.

Drug Administration and Treatment Protocol

FTY720 was used in two dosage regiments (0.5 mg/kg/d, n = 6; 3 mg/kg/d, n = 5). FTY720 was dissolved in sterile saline solution and administered intraperitonealy once a day starting 2 days before transplantation until mice were euthanized. Controls were treated with saline only (n = 6). Giemsa stained blood smears verified the efficiency of FTY720-induced lymphopenia in peripheral blood in trial experiment as previously described (*32, 36, 37*). FTY720 was kindly provided by Novartis (Switzerland).

Orthotopic Corneal Transplantation

The procedure was performed as described previously by She et al. (*44*), with some modifications. Briefly, a 2 mm trephine was used to mark the donor cornea and a sharp needle was used to enter the anterior chamber. To complete the dissection micro scissors were used and the graft was stored in balanced salt solution at room temperature until transplantation. Recipients were anesthetized

with a mixture of Ketamin (Vetalar TM V 100 mg/ml, Pharmacia & Upjohn Ltd., UK, 2.67 mg/mouse) and xylazin (Rompun 2%, Bayer, Germany, 0,267 mg/mouse) intraperitonealy. Mydrum gtt. oph. (tropicamidum 5 mg, Ankerpharm, Germany) and Neo-Synephrine 10% gtt. (phenylephrine hydrochloride 100 mg, Sanofi, Chicago, USA) were used to perform the mydriasis. The corneal graft was fixed in place by interrupted 11/0 Ethilon black monofilament non-absorbable suture (Johnson & Johnson Intl., UK). At the end of the procedure, an antibiotic ointment (Polyfax Ophthalmic ointment (polymyxin B sulphate and bacitricin zinc, Dominion Pharma Ltd, UK)) was applied. Sodium Hyaluronate (10 mg/ml) was used in the course of surgery to protect the corneal endothelium. The sutures were left in place for the duration of the experiment. Grafts were examined daily from 3 to 4 days post transplantation under the operating microscope and mice were sacrificed at the times points stated in Results. The opacity of grafts was assessed according to the following grading score: grade 0, clear graft; grade 1, minor opacity with the structure of the anterior chamber clearly distinguishable; grade 2, opaque stroma with the vessels of iris and pupil visible; grade 3, opaque stroma with at least part of the pupil visible and grade 4, opaque stroma, with undistinguishable structures in the anterior chamber. Opacity higher than 1 was considered as the onset of rejection. One mouse developed a cataract and was excluded from the study. In a preliminary study, we observed a higher incidence of severe lung oedema after induction of anaesthesia (usually after 10 to 60 minutes) when mice were treated with FTY720 shortly beforehand. This adverse effect was most pronounced in the high dose treatment group, but was generally avoided by introducing an interval of several hours between drug administration and the induction of anaesthesia. This adverse effect was only observed in FTY720 treated animals.

Immunohistology

Eyes were removed after the mice were sacrificed and snap frozen in OCT in a liquid nitrogen / Iso-Pentane bath. Eight µm sections were fixed in acetone and then treated with a mixture of Fc-bloc (Rat anti mouse CD16/32, Pharmingen Becton Dickinson Co.) with mice, rat or fetal calf serum to reduce non-specific staining. The following panel of monoclonal antibodies from Pharmingen Becton Dickinson Co. was used in this study: biotinylated hamster anti-mouse CD11c, CD3 epsilon (T3), biotinylated mouse anti-mouse I-A^d, H-$2D^d$, biotinylated rat anti-mouse CD19 and F4/80 (Serotec, UK.) followed by biotinylated anti-hamster IgG (cocktail) (Pharmingen Becton Dickinson Co) when hamster primary antibody was used. Controls included the use of appropriate isotype controls. Sections were stained with an alkaline phosphatase conjugated Strept ABComplex (DAKO A/S, Denmark) and alkaline phosphatase activity visualized using Fast Red TR salt (Sigma Chemical Co., USA).

Immunohistological analysis was performed at two time points corresponding to early (day 9) and late (on average day 12) phase of rejection for control group and days 9 and 21 - 27 (covering decline in rejection (opacity) in low FTY720 regiment group and peak phase of rejection in high FTY720 regiment group). Results obtained using low and high dose treatment protocols did not differ significantly and are combined to give one FTY720 "treated" group. Anterior chamber (AC) cellularity was evaluated using the following grading scheme: grade 0, no free cells in AC; grade I, single cells attached to corneal endothelium; grade II, up to 5 layers of infiltrating cells covering the endothelium; grade III, gross hypercellularity exceeding grade II. Coverage of the graft by mouse epithelium, which is presented as a percentage of the length of the graft, was determined by Zeiss LSM Image Browser software Version 3,2,0,70. Numbers of cells in the

recipient cornea and the graft were determined in either one (or in the case of sections stained for CD19 two) field(s) under 400x magnification within the central area of the graft and in the mid-region of the recipient cornea remote from the sutures.

Evaluation of Draining Lymph Nodes

On day nine mice were sacrificed and ipsilateral submandibular and superficial cervical lymph nodes were explanted under the operating microscope with special care that all tissue surrounding these organs was precisely removed with microsurgery instruments. Organs were stored in sterile saline solution on ice and briefly dried on filter paper before weighting on Mettler AE-163 Analytical balance.

Flow Cytometry Analysis of the Draining Lymph Nodes

Flow cytometric analysis of pooled ipsilateral submandibular (SMLN) and cervical superficial (CSLN) lymph nodes cells was performed on day 9 using single cell suspensions prepared from transplanted control (saline treated) and low-dose (0.5 mg/kg/d FTY720) treated mice, as well naïve non-transplanted controls and FTY720 treated non-transplanted groups of mice (n = 4). Single cell suspensions were obtained by gently pressing the organs through a plastic 70-µm sieve. Cells were treated for 20 minutes on ice in the dark with Fc-Bloc (Rat anti mouse CD16/32, Pharmingen Becton Dickinson Co.) in staining buffer (1% FCS and 1% NaN_3 in PBS) and then stained with the following monoclonal antibodies in staining buffer for 30 minutes; FITC-labeled anti-mouse CD40, FITC-labeled anti-mouse CD86, PE-labeled mouse anti-mouse I-A^d, APC-labeled anti-mouse CD11c, APC-labeled anti-mouse Gr-1, PE-Cy7-labeled anti-mouse CD8a, APC-C-labeled anti-

mouse CD45R/B220 and appropriate isotype controls (BD Biosciences Pharmingen). After washing, cells were fixed with CellFIX™ (BD Biosciences Pharmingen) for a further 30 minutes, washed, and analyzed using a LSR Flow Cytometer (Becton Dickinson Co.). Data were analyzed using FlowJo (4.5.2) software.

Statistical Analysis

The Kaplan-Meier test was used in order to determine the statistical significance of delaying rejection of corneal grafts or unpaired t-test or Mann-Whitney test for immunohistological and flow cytometry data. Probability values below 0.05 were considered statistically significant.

RESULTS

Treatment with FTY720 Significantly Delays and Attenuates Clinical Signs of Xenografts Rejection

Saline-injected mice started to reject their grafts (opacity > 1) on day 8 with maximum pathology primarily at the graft margin. At this time, the grafts of FTY720 treated mice were still completely clear and detectable signs were restricted to a minimal dilatation of vessels in the iris together with a limited neovascularization of the recipient cornea. However, despite continued treatment graft rejection was observed to start between days 11 to 13 in the low dose group, and days 11 to 19 in the high dose group. This delay in the onset of rejection was statistically significant for both treatment groups compared to the controls ($p=0.0016$ in both cases), as well as between treatment groups ($p=0.02$) (Fig. 1).

However, graft rejection in the treated groups was incomplete and transient in some cases. Whereas all grafts in the control group reached Grade 4 opacity by day 11-13, no graft from the treated groups reached this degree of corneal opacification, and after some days, the grafts in the low dose group regained their clarity (22 - 24 days after transplantation) (Fig. 2). In fact, only one graft in the FTY720 treated group reached opacity 4 (opaque stroma with no view of iris detail) during the course of the study, in contrast to 100% of animals in the control group which reached Grade 4. In addition, FTY720 treated grafts retained their shape and showed significantly less signs of tissues separation (sloughing) at the graft/recipient corneal interface (Fig. 3), a clinical sign that was prominent in control mice.

FTY720 reduces the inflammatory response in the grafted eye and allows physiological replacement of the corneal epithelium by cells of host origin.

In order to investigate its mode of action, we analyzed the effect of treatment with FTY720 on the local inflammatory response by immunohistology at two time points: one corresponding to the early (day 9) phase of rejection of controls and, for comparison, the same time point in animals on the low dose regimen which were not showing signs of clinical rejection at this time (corneal opacity <1). In addition, in order to assess the extent and cellular composition of infiltration during ongoing rejection, we analyzed the late phase of rejection for the control group (approximately day 12) and the FTY treated groups (days 21 – 27). The results obtained using low and high dose treatment protocols did not differ significantly and are combined to give one FTY720 "treated" group.

Nine days after transplantation, the onset of rejection was accompanied by a pronounced inflammatory response in the grafted eye of control mice. Large numbers of cells were present in the anterior chamber and significant numbers of lymphocytes and cells of the monocyte/macrophage lineage invaded the surrounding tissue and, to a lesser extent, the graft. Treatment with FTY720 significantly suppressed these inflammatory changes at this time, reducing the numbers of T cells (CD3+), B cells (CD19+) and monocyte/macrophages in the anterior segment (Fig. 4 A, C1-C5). The suppression of lymphocytic infiltration was maintained in the treated mice, but in the later phase of rejection, the numbers of monocyte/macrophages were actually higher in the grafts of the treated animals than controls. (Fig. 4 C4 - 5). This correlated with the separation (sloughing) of the xenograft from the recipient cornea in the control group (Fig. 3, arrows). In contrast, the graft remained in situ in FTY720 treated group, despite the considerable inflammatory response mainly in anterior (subepithelial) part of the stroma.

The suppression of graft rejection by treatment with FTY720 was accompanied by a significant reduction in pathological changes affecting the

corneal epithelium (Fig. 4 B1-2). Immunohistology showed that in the early phase after transplantation the epithelium of the cornea in saline-injected animals had an irregular surface and was considerably thinned. At later stages, the epithelium was mostly absent from the corneal surface, leading to a persistent ulcer (Figures 3 and 4 B1-2). In contrast, the graft epithelium of FTY720-treated mice seemed structurally intact both at 9 days and at later stages, but staining with species-specific antibodies demonstrated that epithelial coverage of the rat xenograft was derived from the recipient i.e. the rat xenograft epithelium had been replaced entirely by healthy, host mouse epithelium even before signs of clinical rejection (stromal opacification) developed at nine days (Fig. 4).

Macroscopic and Cellular Changes in Draining Lymph Nodes Following Corneal Xenograft are Substantially Modified after Treatment with FTY720

Transport of antigen to the submandibular draining lymph node (SMLN) is required for priming the immune response and eventual rejection of corneal allografts in the mouse (*45, 46*). Similar events seemed to occur after xenograft transplantation since there was a significant increase in SMLN size (weight) and cellularity (Table 1). Treatment with FTY720 inhibited these changes (Table 1). Rejection was associated with a three-fold increase in the weight of the SMLN by day 9-post transplantation and this increase was completely blocked by treatment with FTY720 ($p=0.03$) (Table 1).

Flow cytometry revealed that these changes in weight were paralleled by differences in the absolute numbers of cells in the draining lymph nodes (Table 1). In naïve mice, treatment with FTY720 did not significantly influence either the overall ratio of T cells to B cells, or the relative proportions of CD4+, CD8+, CD25+ T cell subsets, but there was an increase in the number of Gr-1+ cells (presumed

granulocytes) when compared with saline-treated control mice. Rejection in sham-injected animals was associated with an increase in the proportion of cells expressing $B220^{HI}$ + (mainly B cells) and CD11c (dendritic cells) relative to the T cell pool, but this was reversed by treatment with FTY720.

In order to evaluate activation of various cells within the draining lymph node we investigated the level of expression of MHC class II, CD40 and CD86 on $B220^{HI}$ + B cells. As anticipated, the expression of class II MHC and CD86 was upregulated within the B cell pool in saline-injected transplanted mice compared to naïve mice, which was indicative of an ongoing immune response. Treatment with FTY720 blocked this response in transplanted mice reducing the levels of expression of these activation markers to below normal levels. Intriguingly, we observed no effect of transplantation or treatment on the expression of these molecules within the CD11c+ population.

DISCUSSION

This study demonstrates that monotherapy with FTY720 significantly delays the onset and severity of rejection in a model of rat-to-mouse xenogenic corneal transplantation, identifying it as an agent that may be valuable in promoting long term survival of corneal xenografts.

FTY720 monotherapy was not previously reported to be particularly effective in delaying xenograft rejection. In the rat, it was reported to only slightly prolong the survival of hamster skin xenografts (*35*) and, although cellular infiltration was reduced in porcine islet xenografts, this did not influence graft survival (*33*). FTY720 brought about no significant improvement in graft survival in other models (*34, 35, 43*), unless used in combination with CsA (*43*), 15-deoxyspergualin (*34*) or FK506 (*32, 35*). The successful modulation of the corneal graft by FTY720 suggests that the mechanism(s) involved in corneal xenograft rejection differ from those involved in the rejection of other organs, as suggested previously [reviewed by Qian (*5*)]. Rejection of xenogeneic corneal grafts is associated with severe inflammation throughout the entire anterior segment. Our results demonstrate that treatment with FTY720, even in monotherapy, substantially delays this inflammatory response in a dose dependent manner and prevents the necrosis at the graft margin and sloughing of the xenograft, possibly enabling graft infiltration at the late time point. Retention of the xenograft enabled coverage of the donor rat stroma by epithelium of mouse origin. Interestingly, this had occurred within 9 days before the graft was clinically rejected. By the fourth week, coverage was usually complete. At that time, immunohistological studies identified only a small number of mouse-derived epithelial cells migrating across the rat graft in control animals, indicating that reepithelialization was initiated, but was ultimately abortive. A previous study of corneal epithelial regeneration in mice similarly reported that syngeneic and

allogeneic epithelium were replaced in 15 days, several days before the onset of clinically apparent rejection (*47*). The results of the present study indicate that the severely inflamed immunologically-rejected xenograft does not support host-derived reepithelialization of the donor xenograft but treatment with FTY720 sufficiently suppresses the severity of the inflammatory response to permit donor graft epithelial coverage by host cells and/or that FTY720 also promotes epithelial growth and migration. This is probably the first step towards permanent engraftment.

Our studies also provide some further insight into the mode of action of FTY720. Previous studies suggest that FTY720 modifies patterns of T cell migration and sequestration (*32-36, 41*). However, we have also found that treatment with FTY720 induces profound reduction in T cell expansion with macroscopically visible prevention of the normal increase in the size of the draining lymph nodes after transplantation and massively reduced cell numbers. This may be due to either reduced T cell activation or proliferation, or increased T cell death in the lymph node prior to emigration to the tissues (activation induced cell death). The associated reduction in B cell expansion and the expression of activation markers (MHC class II and CD86, see Table 1) nine days after transplantation would suggest that FTY720 acts through the former mechanism. Other studies also provide evidence that FTY720 may also have direct effects on the T cell response. For instance, several days' exposure to FTY720 inhibits the T cell response in the MLR (*29, 42, 48, 49*). In vitro this effect is associated with reduced production of IFN-γ and to a lesser extent IL-2 and reduced proliferation of mature T cells in the presence of rhIL-2 (*50*). We cannot exclude the possibility that activation-induced cell death with apoptosis might have occurred extensively during the early stages after priming in FTY720 treated animals. We are currently investigating this mechanism.

The results of this study therefore demonstrate that FTY720, used in isolation, is a potent immunosuppressant in the control of xenogenic corneal graft rejection in the rat-to-mouse model and shows that it maybe possible, at least in experimental treatments, to develop long-term acceptance of corneal xenografts.

Acknowledgment

We would like to thank members of BSU, University of Aberdeen and L. Duncan, Judith Farquhar and M. Blaylock for technical assistance as well as Novartis Institute for Bio Medical Research for synthesis of FTY720.

Figure legends

Figure 1 *Treatment with FTY720 delays the onset of corneal rejection.* All corneas of the control group started to lose transparency and undergo rejection (opacity 1) on day 8. Treatment with either 0.5 or 3.0 mg/kg/d of FTY720 significantly delayed the onset of clinical rejection by 4 (low dose) or 7.6 (high dose) days to 12.0±0.89 and 15.6±3.1 days respectively (p=0.0016).

Figure 2 *Treatment with FTY720 reduces the rate of increase as well as overall extent of corneal opacity after transplantation* Treatment with 0.5 or 3.0 mg/kg/d of FTY720 dose dependently delayed beginning (opacity 1) of rejection and reduced the rate and extent of corneal opacification.

Figure 3 *Representative photographs of cellular infiltration of the anterior segment of the eye during early and late phase after transplantation.* Representative sections of mice eyes stained with anti-mouse MHC I monoclonal antibody. During the late phase the separation of the control graft from the recipient cornea occurred (⇓); there was also a dense organization of cellular infiltrate in the anterior chamber along with mostly absent epithelium (🡻) and, in the FTY720 treated group, the infiltrated graft with bullous keratopathy (∗) covered with epithelium of mouse origin.

Figure 4 *Cellular infiltration of the anterior segment of the eye.* Cellular infiltration was assessed at two different phases after corneal transplantation. In general, treatment with FTY720 significantly reduced cellular infiltration of anterior chamber, grafts, and adjacent tissue initially, but while in the later phase of rejection its effect was most pronounced on lymphocytes, the numbers of monocyte/macrophages were significantly increased in the grafts of FTY720 treated animals. (CO –

transplanted control group treated with saline, LD – transplanted group treated with low dose FTY720, TR – pooled groups of FTY720 (low and high dose) treated mice). Bars representing FTY720 treatment are gray.

Table 1

Flow Cytometry Analysis of Draining Lymph Nodes Cell Populations 9 Days after Transplantation

			Control	Control-TX	FTY720	FTY720-TX
			Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Weight of Organs (mg) (Mean±SD)		**SMLN**	2.55 ± 0.67	7.50 ± 0.98	2.83 ± 1.53	2.53 ± 1.13
		CSLN	1.65 ± 0.44	2.83 ± 0.90	1.90 ± 0.13	2.50 ± 0.70
		Pooled LNs	4.20 ± 1.00	10.30 ± 1.70	4.70 ± 1.60	5.10 ± 1.80
Nr. of Cells/Pooled LNs ($x10^6$) (Mean±SD)		**Nr. of Cells/Pooled LNs ($x10^6$)**	1.39 ± 0.83	8.58 ± 2.82	3.09 ± 0.68	2.76 ± 1.52
		CD3+	1.00 ± 0.58	5.15 ± 1.74	2.16 ± 0.48	1.84 ± 0.98
		CD3+CD4+	0.69 ± 0.39	3.56 ± 1.20	1.46 ± 0.34	1.23 ± 0.65
		CD3+CD8+	0.27 ± 0.16	1.33 ± 0.45	0.58 ± 0.12	0.53 ± 0.29
		CD3--CD25+	0.14 ± 0.08	0.86 ± 0.27	0.34 ± 0.10	0.31 ± 0.17
		$B220^{Hi}$+	0.34 ± 0.20	3.26 ± 1.02	0.84 ± 0.23	0.81 ± 0.47
		CD11c+	0.01 ± 0.01	0.10 ± 0.02	0.02 ± 0.01	0.03 ± 0.02
		GR-1+CD3-	0.04 ± 0.03	0.35 ± 0.12	0.16 ± 0.03	0.15 ± 0.09
Intensity of Fluorescence (Geomean)	**$B220^{HI}$** +	**MHC II+**	38.10 ± 4.36	91.55 ± 17.30	23.13 ± 6.44	25.78 ± 1.89
		CD40+	13.93 ± 1.36	15.20 ± 1.87	11.45 ± 0.33	11.18 ± 0.72
		CD86+	10.00 ± 0.82	17.73 ± 2.92	7.94 ± 0.39	8.75 ± 0.59
	CD11c +	**MHC II+**	73.10 ± 11.51	55.95 ± 8.18	70.93 ± 21.38	56.60 ± 16.51
		CD40+	27.18 ± 1.81	23.98 ± 1.42	27.60 ± 4.28	24.73 ± 4.21
		CD86+	34.33 ± 6.17	33.43 ± 4.39	39.00 ± 4.64	37.35 ± 10.53

Figures

Figure 1

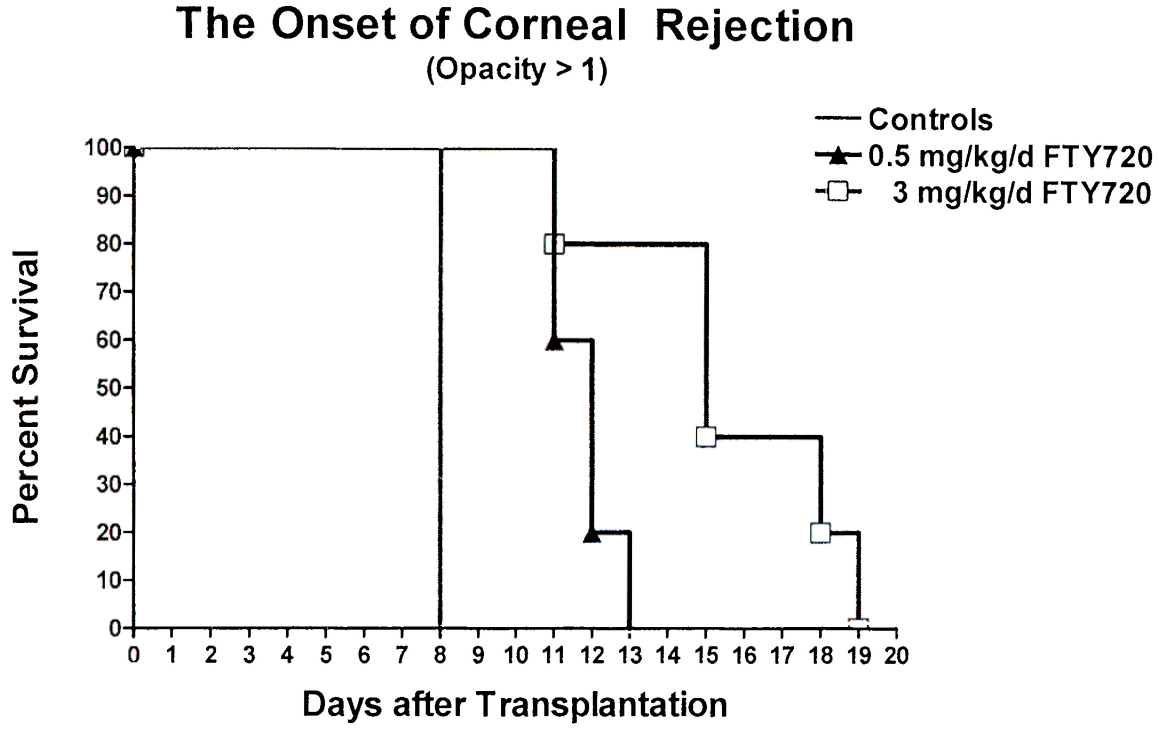


Figure 2

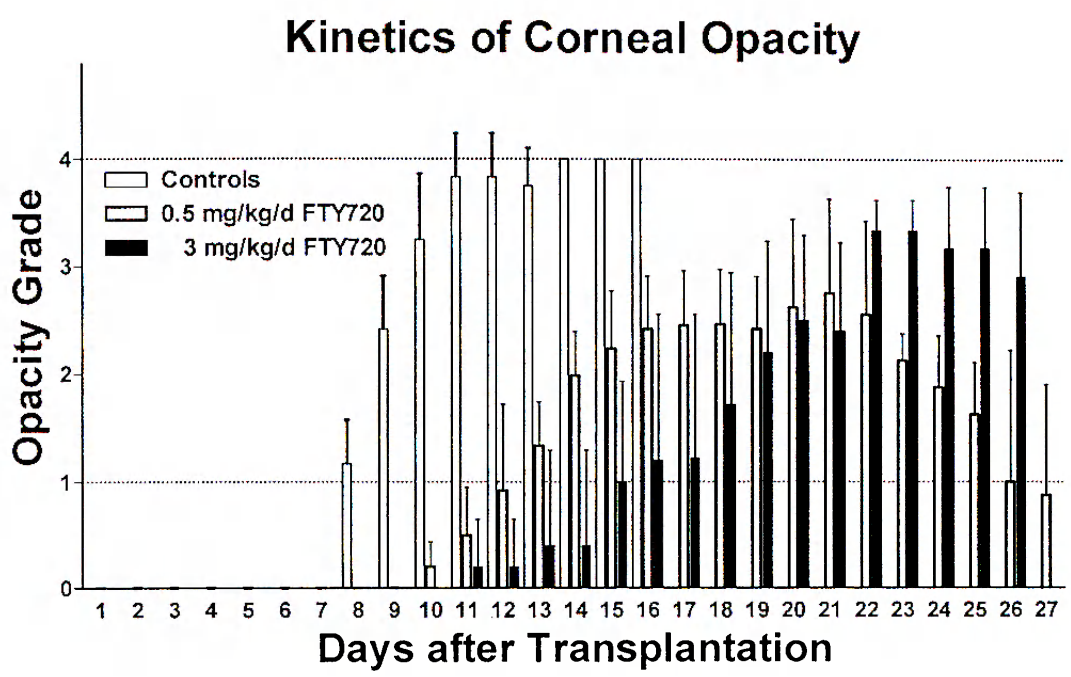

Kinetics of Corneal Opacity
Controls
0.5 mg/kg/d FTY720
3 mg/kg/d FTY720
Opacity Grade
0
1
2
3
4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Days after Transplantation

Figure 3

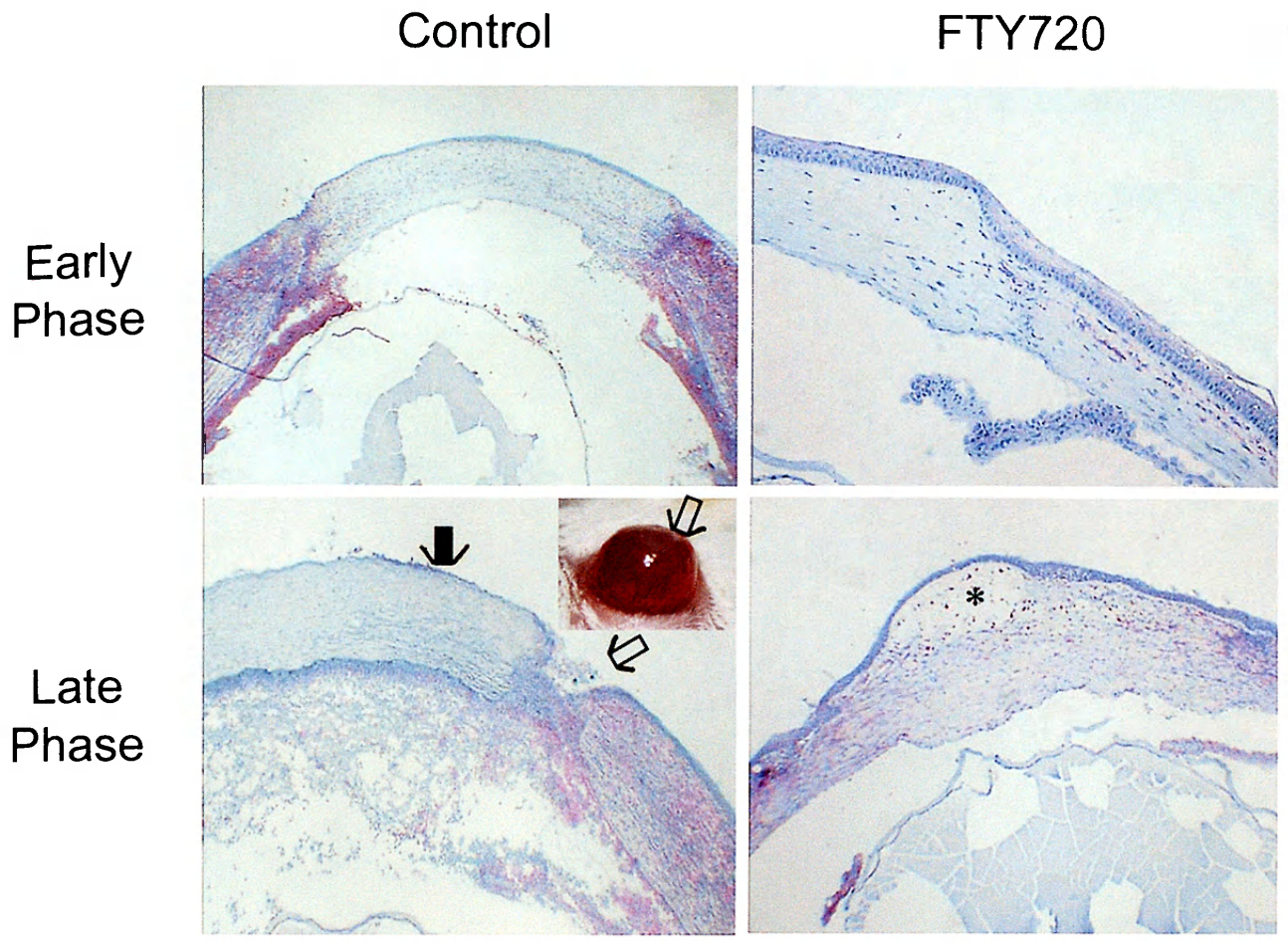
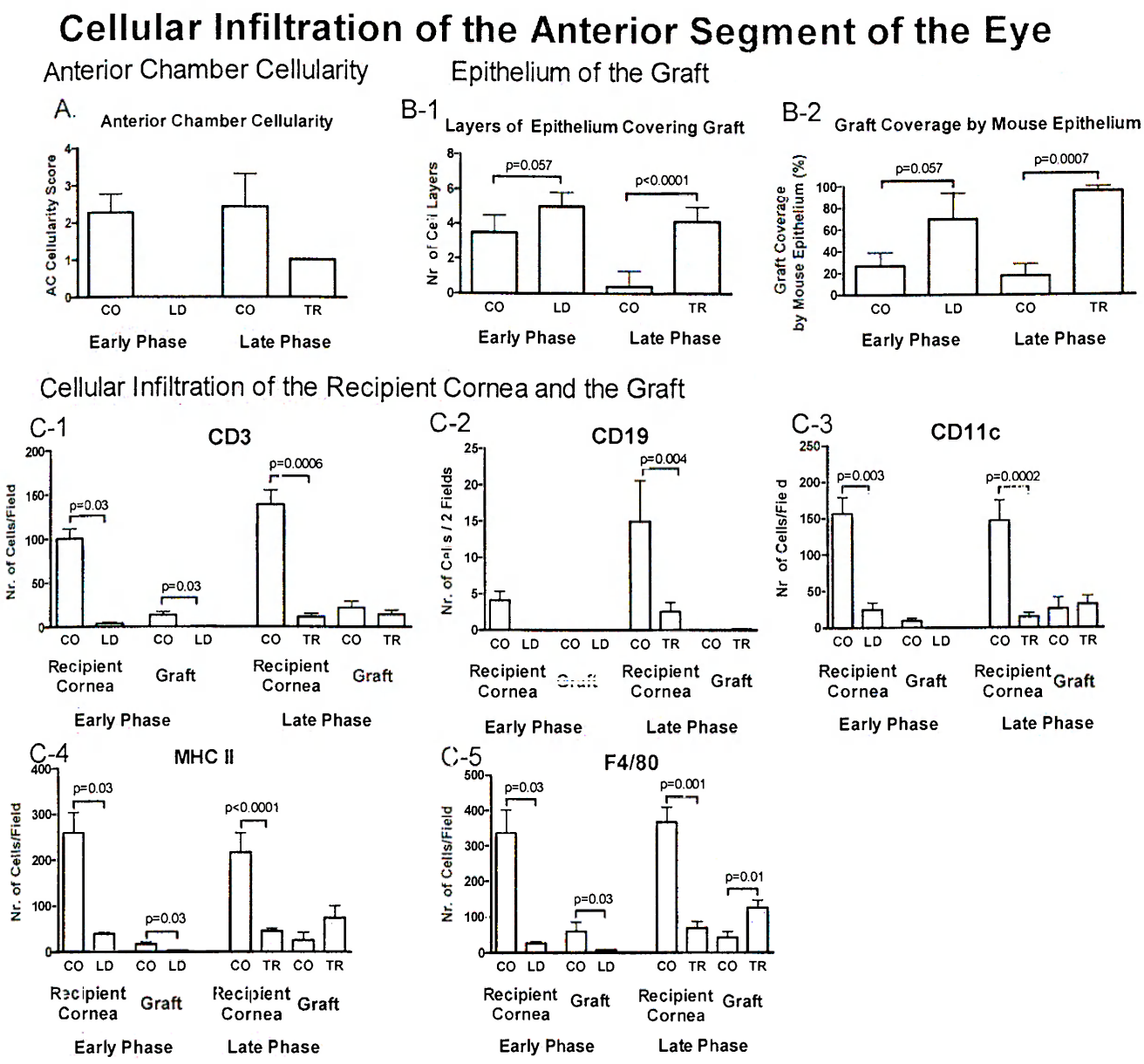


Figure 4


Cellular Infiltration of the Anterior Segment of the Eye
Anterior Chamber Cellularity
Epithelium of the Graft
A.
Anterior Chamber Cellularity
AC Cellularity Score
B-1
Layers of Epithelium Covering Graft
Nr of Cell Layers
p=0.057
p<0.0001
B-2
Graft Coverage by Mouse Epithelium
Graft Coverage by Mouse Epithelium (%)
p=0.057
p=0.0007
CO LD CO TR
Early Phase
Late Phase
Cellular Infiltration of the Recipient Cornea and the Graft
C-1
CD3
Nr. of Cells/Field
p=0.03
p=0.03
p=0.0006
Recipient Cornea
Graft
Early Phase
Late Phase
C-2
CD19
Nr. of Cells / 2 Fields
p=0.004
C-3
CD11c
Nr of Cells/Field
p=0.003
p=0.0002
C-4
MHC II
Nr. of Cells/Field
p=0.03
p=0.03
p<0.0001
C-5
F4/80
Nr. of Cells/Field
p=0.03
p=0.03
p=0.001
p=0.01

REFERENCES

1. The collaborative corneal transplantation studies (CCTS). Effectiveness of histocompatibility matching in high-risk corneal transplantation. The Collaborative Corneal Transplantation Studies Research Group. Arch Ophthalmol 1992; 110 (10): 1392.
2. Williams KA, Roder D, Esterman A, Muehlberg SM, Coster DJ. Factors predictive of corneal graft survival. Report from the Australian Corneal Graft Registry. Ophthalmology 1992; 99 (3): 403.
3. Reinhard T, Bohringer D, Enczmann J, et al. HLA class I and II matching improves prognosis in penetrating normal-risk keratoplasty. Dev Ophthalmol 2003; 36: 42.
4. Khaireddin R, Wachtlin J, Hopfenmuller W, Hoffmann F. HLA-A, HLA-B and HLA-DR matching reduces the rate of corneal allograft rejection. Graefes Arch Clin Exp Ophthalmol 2003; 241 (12): 1020.
5. Qian Y, Dana MR. Molecular mechanisms of immunity in corneal allotransplantation and xenotransplantation. Expert Rev Mol Med 2001; 2001: 1.
6. Yamagami S, Isobe M, Yamagami H, Hori J, Tsuru T. Mechanism of concordant corneal xenograft rejection in mice: synergistic effects of anti-leukocyte function-associated antigen-1 monoclonal antibody and FK506. Transplantation 1997; 64 (1): 42.
7. Tanaka K, Yamada J, Streilein JW. Xenoreactive CD4+ T cells and acute rejection of orthotopic guinea pig corneas in mice. Invest Ophthalmol Vis Sci 2000; 41 (7): 1827.

8. Chitilian HV, Laufer TM, Stenger K, Shea S, Auchincloss H, Jr. The strength of cell-mediated xenograft rejection in the mouse is due to the CD4+ indirect response. Xenotransplantation 1998; 5 (1): 93.

9. Higuchi R, Streilein JW. CD8+ T cell-mediated delayed rejection of orthotopic guinea pig cornea grafts in mice deficient in CD4+ T cells. Invest. Ophthalmol. Vis. Sci. 2003; 44 (1): 175.

10. Larkin DF, Takano T, Standfield SD, Williams KA. Experimental orthotopic corneal xenotransplantation in the rat. Mechanisms of graft rejection. Transplantation 1995; 60 (5): 491.

11. Ross JR, Sanfilippo FP, Howell DN. Histopathologic features of rejecting orthotopic corneal xenografts. Curr Eye Res 1994; 13 (10): 725.

12. Hegde S, Mellon JK, Hargrave SL, Niederkorn JY. Effect of alloantibodies on corneal allograft survival. Invest. Ophthalmol. Vis. Sci. 2002; 43 (4): 1012.

13. Goslings WR, Yamada J, Dana MR, et al. Corneal transplantation in antibody-deficient hosts. Invest Ophthalmol Vis Sci 1999; 40 (1): 250.

14. Hla T. Physiological and pathological actions of sphingosine 1-phosphate. Semin Cell Dev Biol 2004; 15 (5): 513.

15. Paugh SW, Payne SG, Barbour SE, Milstien S, Spiegel S. The immunosuppressant FTY720 is phosphorylated by sphingosine kinase type 2. FEBS Lett 2003; 554 (1-2): 189.

16. Billich A, Bornancin F, Devay P, Mechtcheriakova D, Urtz N, Baumruker T. Phosphorylation of the immunomodulatory drug FTY720 by sphingosine kinases. J Biol Chem 2003; 278 (48): 47408.

17. Sanchez T, Estrada-Hernandez T, Paik JH, et al. Phosphorylation and action of the immunomodulator FTY720 inhibits vascular endothelial cell growth factor-induced vascular permeability. J Biol Chem 2003; 278 (47): 47281.

18. Allende ML, Sasaki T, Kawai H, et al. Mice deficient in sphingosine kinase 1 are rendered lymphopenic by FTY720. J Biol Chem 2004.

19. Brinkmann V, Davis MD, Heise CE, et al. The immune modulator FTY720 targets sphingosine 1-phosphate receptors. J Biol Chem 2002; 277 (24): 21453.

20. Mandala S, Hajdu R, Bergstrom J, et al. Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. Science 2002; 296 (5566): 346.

21. Matloubian M, Lo CG, Cinamon G, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature 2004; 427 (6972): 355.

22. Allende ML, Dreier JL, Mandala S, Proia RL. Expression of the sphingosine 1-phosphate receptor, S1P1, on T-cells controls thymic emigration. J Biol Chem 2004; 279 (15): 15396.

23. Graler MH, Goetzl EJ. The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G protein-coupled receptors. FASEB J. 2004: 03.

24. Suzuki S, Enosawa S, Kakefuda T, et al. Immunosuppressive effect of a new drug, FTY720, on lymphocyte responses in vitro and cardiac allograft survival in rats. Transpl Immunol 1996; 4 (3): 252.

25. Suzuki S, Kakefuda T, Amemiya H, et al. An immunosuppressive regimen using FTY720 combined with cyclosporin in canine kidney transplantation. Transpl Int 1998; 11 (2): 95.

26. Suzuki S, Enosawa S, Kakefuda T, et al. A novel immunosuppressant, FTY720, with a unique mechanism of action, induces long-term graft acceptance in rat and dog allotransplantation. Transplantation 1996; 61 (2): 200.

27. Masubuchi Y, Kawaguchi T, Ohtsuki M, et al. FTY720, a novel immunosuppressant, possessing unique mechanisms. IV. Prevention of graft versus host reactions in rats. Transplant Proc 1996; 28 (2): 1064.

28. Xu M, Antoniou EA, Afford SC, et al. Effect of peritransplant FTY720 alone or in combination with posttransplant FK 506 in a rat model of cardiac allotransplantation. Transplant Proc 1997; 29 (7): 2964.

29. Zhang EP, Muller A, Ignatius R, Hoffmann F. Significant prolongation of orthotopic corneal-graft survival in FTY720-treated mice. Transplantation 2003; 76 (10): 1511.

30. Suzuki K, Yan H, Li XK, Amemiya H, Suzuki S, Hiromitsu K. Prevention of experimentally induced autoimmune type I diabetes in rats by the new immunosuppressive reagent FTY720. Transplant Proc 1998; 30 (4): 1044.

31. Mitsusada M, Suzuki S, Kobayashi E, Enosawa S, Kakefuda T, Miyata M. Prevention of graft rejection and graft-versus-host reaction by a novel immunosuppressant, FTY720, in rat small bowel transplantation. Transpl Int 1997; 10 (5): 343.

32. Miyata Y, Ohdan H, Noriyuki T, et al. Prolongation of concordant xenograft survival by treatment with FTY720. Transplant Proc 1998; 30 (8): 4163.

33. Maeda A, Goto M, Zhang J, et al. Immunosuppression with FTY720 and cyclosporine A inhibits rejection of adult porcine islet xenografts in rats. Transplantation 2003; 75 (8): 1409.

34. Fujiwara I, Nakajima H, Matsuda T, Mizuta N, Yamagishi H, Oka T. Prolongation of concordant xenograft survival by a newly developed drug, FTY720. Transplant Proc 1999; 31 (7): 2831.

35. Fukuda Y, Ohdan H, Miyata Y, Shintaku S, Dohi K. Effect of FTY720 on immunoregulation in concordant xenotransplantation. Transpl Int 1998; 11 Suppl 1: S461.

36. Yagi H, Kamba R, Chiba K, et al. Immunosuppressant FTY720 inhibits thymocyte emigration. Eur J Immunol 2000; 30 (5): 1435.

37. Yanagawa Y, Sugahara K, Kataoka H, Kawaguchi T, Masubuchi Y, Chiba K. FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. II. FTY720 prolongs skin allograft survival by decreasing T cell infiltration into grafts but not cytokine production in vivo. J Immunol 1998; 160 (11): 5493.

38. Ueda H, Takahara S, Azuma H, Kusaka M, Suzuki S, Katsuoka Y. Effect of a novel immunosuppressant, FTY720, on allograft survival after renal transplant in rats. Eur Surg Res 2000; 32 (5): 279.

39. Hozumi Y, Kobayashi E, Miyata M, Fujimura A. Immunotherapy for experimental rat autoimmune thyroiditis using a novel immunosuppressant, FTY720. Life Sci 1999; 65 (17): 1739.

40. Miyamoto T, Matsumori A, Hwang MW, Nishio R, Ito H, Sasayama S. Therapeutic effects of FTY720, a new immunosuppressive agent, in a murine model of acute viral myocarditis. J Am Coll Cardiol 2001; 37 (6): 1713.

41. Chiba K, Yanagawa Y, Masubuchi Y, et al. FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. I. FTY720 selectively decreases the number of circulating mature lymphocytes by acceleration of lymphocyte homing. J Immunol 1998; 160 (10): 5037.

42. Luo ZJ, Tanaka T, Kimura F, Miyasaka M. Analysis of the mode of action of a novel immunosuppressant FTY720 in mice. Immunopharmacology 1999; 41 (3): 199.

43. Zhang J, Song Z, Wijkstrom M, et al. FTY720 in combination with CsA inhibits islet xenograft rejection: a study in the pig-to-rat model. Transplant Proc 2000; 32 (5): 1017.

44. She SC, Steahly LP, Moticka EJ. A method for performing full-thickness, orthotopic, penetrating keratoplasty in the mouse. Ophthalmic Surg 1990; 21 (11): 781.

45. Yamagami S, Dana MR. The critical role of lymph nodes in corneal alloimmunization and graft rejection. Invest Ophthalmol Vis Sci 2001; 42 (6): 1293.

46. Plskova J, Duncan L, Holan V, Filipec M, Kraal G, Forrester JV. The immune response to corneal allograft requires a site-specific draining lymph node. Transplantation 2002; 73 (2): 210.

47. Hori J, Streilein JW. Dynamics of donor cell persistence and recipient cell replacement in orthotopic corneal allografts in mice. Invest Ophthalmol Vis Sci 2001; 42 (8): 1820.

48. Konishi K, Inobe M, Yamada A, Murakami M, Todo S, Uede T. Combination treatment with FTY720 and CTLA4IgG preserves the respiratory epithelium and prevents obliterative disease in a murine airway model. J Heart Lung Transplant 2002; 21 (6): 692.

49. Kurose S, Ikeda E, Tokiwa M, Hikita N, Mochizuki M. Effects of FTY720, a novel immunosuppressant, on experimental autoimmune uveoretinitis in rats. Exp Eye Res 2000; 70 (1): 7.

50. Hwang MW, Matsumori A, Furukawa Y, et al. FTY720, a new immunosuppressant, promotes long-term graft survival and inhibits the progression of graft coronary artery disease in a murine model of cardiac transplantation. Circulation 1999; 100 (12): 1322.

Chapter 7

FTY720 in Corneal Allotransplantation

Klára Sedláková, Marie Robertson, Elizabeth Muckersie, Linda Duncan,

Martin Filipec, Vladimír Holáň, and John V. Forrester

In preparation

FTY720 in Corneal Allotransplantation

Klára Sedláková[1,2], Marie Robertson[1], Elizabeth Muckersie[1], Linda Duncan[1], Martin Filipec[3,4], Vladimír Holáň [5,6], and John V. Forrester[1,*]

[1]Department of Ophthalmology, IMS, University of Aberdeen, Scotland, UK

[2]Department of Ophthalmology, 1st Medical Faculty of Charles University and General Faculty Hospital, Prague, Czech Republic

[3]Laboratory of Ophthalmology and Eye Bank, 1st Medical Faculty and General Faculty Hospital, Charles University, Prague, Czech Republic

[4]Eye Clinic Lexum, Prague, Czech Republic

[5]Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic

[6]Faculty of Natural Sciences, Charles University, Prague, Czech Republic

Running Title: FTY720 in Corneal Allotransplantation

Key words: Corneal transplantation, FTY720, Lymph node, Dendritic cell, B cell, MHC class II.

***Address correspondence to:** J.V. Forrester, Department of Ophthalmology, IMS, University of Aberdeen, Medical School, AB25 2ZD, Scotland, UK. Tel.: +44 1224 553782, Fax: +44 1224 555955, e-mail: j.forrester@abdn.ac.uk

Abstract

Background. FTY720 is an effective immunosuppressant in various experimental transplantation models. The modification of cell traffic and egress of mature lymphocytes was suggested as the main mode of its action. Here we show that FTY720 suppresses also early graft infiltration by CD11+ cells and consequent T and B cell priming.

Methods. Mice were grafted orthotopically with allogeneic corneas and the recipients were treated with FTY720 (or saline) using various pre-, per- or post-graft regimes. Graft survival was assessed clinically and the changes in the grafts or in draining lymph nodes were evaluated.

Results. FTY720 significantly delayed corneal graft rejection using various pre-, per- and post-graft regimens. No graft developed signs of rejection while the drug was administered. However, rejection occurred almost uniformly, 10-17d after drug withdrawal. Postponing treatment for 6d after transplantation did not compromise its beneficial effect. Corneal histology at 2.5d after transplantation showed a significant reduction in CD11c+ cell infiltration into the cornea of FTY720-treated mice, despite a pronounced infiltration by other cell types, particularly macrophages. In contrast, FTY720 markedly suppressed later (20d) cellular infiltration into the cornea and led to reduced cell numbers and activation status of B cells in the draining lymph nodes.

Conclusion. FTY720 prevents corneal allograft rejection when continuously administered and suppresses graft infiltration and lymphocyte priming. These effects may be linked to early reduction in recruitment of bone marrow derived CD11c+ dendritic cells to the graft and consequently by limiting both T and B cell priming and expansion in the draining lymph nodes.

Immunological rejection is the principal cause of the corneal graft failure (1). Clinically, systemic immunosuppression is increasingly employed to control rejection especially in high-risk cases (2). FTY720 prevents graft rejection in several experimental models, such as skin (3), heart (4), liver (5) or small bowel grafts (6) transplantation. Importantly, the addition of FTY720 allows a decrease in CsA dose, reducing its adverse effects (7). FTY720 is also effective as an immunosuppressant in models of autoimmunity (6, 8-11).

Corneal graft rejection occurs mainly via the "indirect" pathway involving a CD4+ T cell mediated response to alloantigens (12). FTY720 delays corneal allograft rejection in mice in combination with dexamethasone (13) and is effective also in concordant xenograft transplantation (14). Recently, FTY720 monotherapy was used in a rat corneal allograft model, where it differentially modified infiltration of CD4 and CD8 T cells into the graft (15).

The main mode of FTY720 action in the lymphoid system is thought to be sequestration of T and B cells in lymph nodes and Peyer's patches (16). Facilitation of lymphocyte homing across high endothelium venules (17) and inhibition of egress of mature lymphocytes from the peripheral lymphoid organs and the thymus (18) (where a reduction in CD4+8+TCR$\alpha\beta$- thymocytes results in their impaired differentiation and maturation (19)) together contribute to a systemic lymphopenia (20). Seemingly, the loss of responsiveness toward plasmatic S1P and downregulation of S1P1 receptors is responsible for inhibition of egress to the circulation (21). The S1P receptor is a master regulator of T cell traffic from the thymus and the lymph nodes, and FTY720 induces a similar but temporary S1P1 null state (18). Other mechanisms of FTY720 action are also involved. For instance, induction of apoptosis in T and B cells shortly after FTY720 administration has been reported both in vivo and in vitro (22, 23). In the periphery, FTY720 preserves

vascular integrity by enhancing adherence junction assembly and barrier function of endothelial cells (24), thus limiting inflammation-induced vascular permeability transendothelial cellular infiltration (25). Similarly, it increases formation of endothelial junctional complexes and parenchymal cell-to-cell contacts in lymph nodes (26).

In this study, we evaluated effects of FTY720 on priming and activation of immune cells after corneal allotransplantation. FTY720 prevented corneal allograft rejection during its administration by mediating a profound reduction in T and B cell expansion and progressive limitation of cell number in the draining lymph node (DLN). Intriguingly, improved graft survival was associated with a reduction in the early infiltration of CD11c+ presumed dendritic cells (DC) to the host cornea, suggesting a defect in DC recruitment to the graft and reduced T and B cell priming.

MATERIALS AND METHODS

Animals

Female C57Bl/6 mice (10- to 18-week-old) were used as recipients and BALB/c mice as donors. Animals were obtained from the Biological Services Unit, University of Aberdeen and handled in accordance with the UK Animal License Act 1986 and ARVO Statement for the Use of Animals in Ophthalmic and Visual Research.

Drug Administration and Treatment Protocol

FTY720, kindly provided by Novartis (Basel, Switzerland), in sterile saline solution was administered daily for a defined period at a dose 0.5 mg/kg intraperitonealy. Control mice were treated with sterile saline only. To evaluate effects of FTY720 during different phases of the immune response mice were treated for five different time periods: -2 to 6d (n=7), -2 to 20d (n=7), 2 to 12d (n=5), 6 to 12d (n=5), 6 to 20d (n=5) after transplantation and followed until signs of corneal graft rejection were present. For experiments involving weighing of lymph nodes, cell counting and flow cytometry analysis, FTY720 (or saline) were given daily starting 2d before transplantation and continued until 2.5, 6, 12 or 20d and from 6 to 12d after transplantation.

Orthotopic Corneal Transplantation

Recipients were anesthetized with a mixture of ketamin (Vetalar, Pharmacia & Upjohn Ltd., Northants, UK) and xylazin (Rompun inj. solution, Bayer, Leverkusen, Germany) intraperitonealy. Tropicamidum (Mydrum, Ankerpharm GmbH, Berlin, Germany) and phenylephrine hydrochloride (Neo-Synephrine, Sanofi, Chicago, IL) were used to induce mydriasis. The corneal graft (∅ 2 mm)

was fixed in place by interrupted 11/0 Ethilon black monofilament non-absorbable sutures (Johnson & Johnson Intl., Yorkshire, UK) and an antibiotic ointment (Polyfax Ophthalmic ointment, Dominion Pharma Ltd, Haslemere Surrey, UK) was applied. Sodium hyaluronate (10 mg/ml) was used during the surgery to protect the corneal endothelium. Grafts were examined at least three times a week or daily when rejection was ongoing and mice were sacrificed at the time points stated in Results. Grafts were evaluated clinically for rejection by grading corneal opacification as follows: Grade 0, clear graft; grade 1, minor opacity with the pupil margin clearly distinguishable; grade 2, opaque stroma with the pupil margin visible; grade 3, opaque stroma with at least part of the pupil visible and grade 4, opaque stroma, with undistinguishable structures in the anterior chamber. The "day of the rejection" was set as the day when the opacity of the cornea increased to the extent that only the pupil margin remained distinguishable (opacity 3).

Immunohistology

Immunohistological analysis was performed at two time points: (a) 2.5d post graft (eight grafts in both groups) and (b) the day when control grafts were considered rejected, which corresponded with the 20d time point in mice treated daily with FTY720 from 2d before transplantation (n=5 and 7) (see Results).

Eyes were snap frozen in OCT in liquid nitrogen / Iso-Pentane and sections (10 μm) were fixed in acetone. The following panel of monoclonal antibodies were used: biotinylated hamster anti-mouse CD11c, CD3ε (T3); biotinylated mouse anti-mouse I-Ad, biotinylated rat anti-mouse CD11b, biotinylated rat anti-mouse F4/80 or rat anti-mouse F4/80 (Serotec, Oxford, UK) and APC-conjugated rat anti-mouse Ly-6G (GR-1) and Ly-6C (RB6-8C5) [BD Biosciences-Pharmingen, Oxford, UK (BD)] followed by rabbit anti-rat secondary antibody

(DakoCytomation Denmark A/S, Glostrup, Denmark). Controls included isotype IgGs. Sections were stained with an alkaline phosphatase conjugated Strept ABComplex (DakoCytomation Denmark A/S) and alkaline phosphatase activity visualized using Fast Red TR salt (Sigma Chemical Co., St. Louis, MI). The numbers of cells in two optical fields of the recipient cornea and the graft remote from the sutures were determined under 400 x magnifications.

Evaluation of DLN

At specified times mice were sacrificed and ipsilateral submandibular (SMDLN) and superficial cervical lymph nodes (CSLN) were explanted from individual mice (n = 4 to 12 per group). Surrounding tissues from these organs was carefully removed and the nodes were weighed. Single cell suspensions were then prepared and counted. Flow cytometric analysis of pooled SMDLN and CSLN cells was performed individually for each mouse from the following groups: (a) transplanted control "Co-TX" (saline treated), (b) FTY720 treated transplanted mice "FTY-TX", (c) naïve non-transplanted controls "Co" and (d) FTY720 treated non-transplanted "FTY" mice at days: 2.5, 6, 12 and 20. Experiments were repeated 2-3 times. Cells were treated for 20 minutes on ice in the dark with Fc-Bloc (rat anti-mouse CD16/32, BD in staining buffer (1% foetal calf serum plus 1% NaN3 in PBS) and then stained with the following monoclonal antibodies in staining buffer for 30 minutes; FITC-labelled anti-mouse CD40, FITC-labelled anti-mouse CD80, FITC-labelled anti-mouse CD86, PE-labelled mouse anti-mouse I-Ad, APC-labelled anti-mouse CD11c, PE-Cy7-labeled anti-mouse CD8a, APC-C-labelled anti-mouse CD45R/B220 and isotype controls (all from BD). After washing, cells were fixed with CellFIX™ (BD) for a further 30 minutes, washed again and analyzed using a LSR Flow Cytometer (BD). Data were analyzed using FlowJo (4.5.2) software.

Detection of Apoptosis

Apoptosis was determined on frozen sections (12 μm) of draining (SMDLN) and non-draining (axillary) lymph nodes on day 20 after transplantation in mice treated daily with saline (n=4 to 5) or FTY720 (n=6 to 7) starting two days before transplantation.

The ApopTag® Fluorescein In Situ Apoptosis Detection Kit (Intergene Company, Purchase, NY) was used according to the manufacturer's instructions. After applying anti-digoxigenin conjugate, sections were washed in Tris-buffered saline, treated with a mixture of Fc-Bloc (rat anti-mouse CD16/32, BD) and mouse serum and incubated with alkaline phosphatase-IgG fraction monoclonal mouse anti-fluorescein (FITC) antibody. Alkaline phosphatase activity was visualized using Fast Red TR salt (Sigma Chemical Co., St. Louis, MI). Sections were then counterstained with Harris′ haematoxylin and mounted. As a negative control, sections were stained identically with TdT ENZYME reagent substituted by deionised water as suggested by the manufacturer. Thymus sections served as a positive control.

TUNEL-positive cells per section were counted under 400x magnification. Sections were then photographed using a ProgRes C14 high-resolution camera (JENOPTIK Laser, Optik, Systeme GmbH, Jena, Germany) (all under 25x magnification) and the area of each section was determined by Zeiss LSM Image Browser software. The final number of TUNEL-positive cells per unit area (approx. 1.44 μm2) was calculated as the number of cells per section divided by the area of section.

Statistical Analysis

The Kaplan-Meier test was used to determine statistical significance of clinical data on corneal graft rejection, unpaired t-test for immunohistological data or unpaired t test with Welch's correction for analysis of apoptosis data in lymph nodes. Mann-Whitney test was used for weight, cellularity, and flow cytometry data. Probability values below 0.05 were considered statistically significant.

RESULTS

Continued Administration of FTY720 is Required to Prevent Corneal Graft Rejection

FTY720 was administered in five groups of C57BL/6 recipient mice for different periods of time covering separate phases of the immune response after transplantation of corneas from BALB/c mice (-2 to 6d, -2 to 20d, 2 to 12d, 6 to 12d, 6 to 20d) and the mice were followed for signs of graft rejection.

Saline treated recipients rejected the allografts at a median of 16.5d. All 5 regimens of FTY720 treatment significantly ($p < 0.001$) delayed rejection compared to saline treated controls (Fig. 1A) while the drug was continuously administered. However, further analysis showed that the interval to graft rejection after drug withdrawal was not significantly different for each of the treatment regimens (median time of 10 to 17d) (Fig. 1B). Furthermore, delaying FTY720 treatment to 2 or 6d after transplantation did not significantly limit its effect on survival of the grafts.

FTY720 Limits Early Infiltration of CD11c+ Cells into Grafted Corneas

We next evaluated the effect of FTY720 on initial (2.5d) and late (20d) cellular infiltration of grafted eyes in mice treated daily from two days before transplantation (-2d). Immunohistological analysis revealed that FTY720 did not significantly affect overall early cellular infiltration (Fig. 2A) which was mainly CD11b+ monocyte/macrophage cells. However, there was a 30% reduction ($p < 0.01$) in CD11c+ cells at this time. FTY720 treated mice also had limited corneal neovascularization, similar to that observed following syngeneic transplantation (Fig. 2B). Cellular infiltration in the host was significantly greater than in the donor cornea at this time. Twenty days after transplantation, monocyte/macrophages, T cells and Gr-1 + cells (presumed granulocytes) heavily infiltrated grafts in the

control saline-treated group (Fig. 2C). Cellular infiltration at this time was higher in the donor graft than in the recipient cornea, and the overall level of inflammation was greater than at 2.5d (see Figs. 2A and C). However, in FTY720 treated mice, inflammatory cell infiltration of all cell types was almost completely suppressed ($p < 0.001$) at this time.

FTY720 Suppresses the Cellular Response in DLN and Significantly Reduces Expression of MHC Class II Molecules on B220+ Cells after Transplantation

To investigate the mode of action of FTY720 on the cellular immune response, we analysed changes in the DLN (SMDLN) and compared them with the adjacent non-DLN (CSLN) by harvesting lymph nodes at different times after grafting and/or after starting of treatment with FTY720. We compared lymph node weight, total cell counts and cell subpopulations at four time-points corresponding to the different phases of the immune response: 2.5, 6, 12, and 20d. No change in weight was observed in lymph nodes harvested from control or treated mice 2.5 or 6d after grafting (Fig 3A and B). In the control, saline-treated grafted mice an increase in weight and cellularity of the SMDLN was observed only after 6d (see Fig. 3A and Table 1). However, such increase in weight and cellularity of the SMDLN nodes was not observed in mice receiving FTY720 treatment. These effects were restricted to the site-specific SMDLN (Fig. 3A) and were not observed in the adjacent CSLN (Fig. 3B). Delaying FTY720 treatment for 6d after transplantation still significantly reduced the weight of the DLN compared to the grafted control group at 12d (Fig. 3A). Interestingly, we observed at 20d that in FTY720 treated mice the weight (Fig. 3A) and cellularity (Table 1) of the DLN significantly ($p<0.001$) dropped below the level of control mice (3 separate experiments). Although FTY720 induced profound lymphopenia in the blood (data

not shown), we did not detect a substantial increase in the numbers of lymphocytes in any of tested lymph nodes from FTY720 treated animals.

We were interested to observe the early phenotypic changes in cells in the DLN since we have previously shown that changes in cell subpopulations occur very early after the placement of the corneal graft (27). Analysis of cell populations in DLN indicated that the significant change in weight in the lymph nodes of control mice 12d after transplantation was due to an increase in the total cell count, mainly accounted for by increases in both CD3+ and B220+ populations (Table 1, see for instance column 3). Interestingly, the B220+ population in control-grafted mice expressed high levels of MHC Class II molecules that was significantly reduced after FTY720 treatment. These changes were observed as early as 48h after initial drug administration (the earliest time point evaluated in vivo; data not shown) and remained unchanged throughout the treatment period (Table 1, Fig. 4A). This effect was most prominent on B220+CD40+ cells, i.e. the population with the highest level of MHC class II expression on the surface when compared with cells positive for CD80 or CD86. Stimulation by LPS in vitro partially restored MHC class II expression but significantly below that of similarly treated control cultures. In addition, we could not detect any consistent pattern of effect of FTY720 on the expression of MHC class II on CD11c positive cells (data not shown).

As indicated above, DLN of mice treated with FTY720 20d after transplantation appeared to demonstrate up to 55% reduction in cellularity (and weight) compared to controls (Fig. 3, Table 1). We therefore tested the possibility that cells from DLN are deleted by staining appropriate sections for apoptosis.

As demonstrated in Fig. 5, there were no significant changes in TUNEL-positive cells between DLN cells from control (saline-treated) and FTY720-treated mice 20 days after transplantation. Similarly, no differences were seen in a number

of TUNEL-positive cells in non-draining lymph nodes from control and FTY720-treated mice (Fig. 5). These data suggest that if apoptosis of lymph node cells contributes to cellular drop it happens predominantly before day 20.

DISCUSSION

The data from the current experiment demonstrate that monotherapy with FTY720 at a dosage as low as 0.5 mg/kg/d prevented corneal graft rejection, but the drug was only effective if administered continuously. Clinically, corneas in treated mice were clear at the time of drug withdrawal with only minimal neovascularization at 20d. Treatment with FTY720 significantly reduced early infiltration of the grafted eye with CD11c+ cells while having only a little effect on other cell populations. However, a considerable reduction in all immune cell populations, particularly CD3+ T cell (up to 100 % reduction), was observed 20d after transplantation in FTY720-treated mice. These data suggest that FTY720 might selectively limits the early CD11c+ (presumed) DC recruitment to the graft, with probable downstream effects on T and B cell activation in the DLN, and impairment of the allospecific T cell and effector macrophage responses. This notion gain support from recent data indicating that FTY720 treatment induces retention of DC in the blood stream by preventing transendothelial migration and entry of DC into the tissues (28).

Our results thus provide additional insight to the mode of action of FTY720. FTY720 has been shown to modify the pattern of T cell migration and sequestration in DLN and the thymus (29-31). Our data demonstrate that FTY720 markedly restricts the expansion of T and B cells in DLN that is normally triggered by transplantation. The failure of DC to traffic through the graft and transport sufficient levels of alloantigen to the DLN may contribute to reduced T and B cell activation and proliferation in the DLN but other mechanisms may be involved, since postponing the treatment with FTY720 to 6 days after transplantation also limits T and B cellular expansion in DLN.

T cell proliferation is induced in corneal graft rejection predominantly by the indirect mode of allorecognition (32) in which donor MHC allo-peptides and/or minor histocompatibility antigens are presented in the context of host MHC class II molecules (11, 29). Antigen presenting cells include DC, macrophages and B cells and while the first of these are likely to be involved in the initiation of the graft response, continued presentation of alloantigen may possibly involve presentation of allopeptide or minor antigens by other antigen presenting cells. The immunostimmulatory capacity of DC, and their ability to traffic, is impaired by FTY720 (28, 33). B cells that express high levels of MHC class II on their surface are also effective antigen presenting cells (34). Recent studies of the antigen presenting function of B cells have highlighted their essential role in sustaining the immune response after initial induction by DC (35). In particular, memory T cells, which are likely to be involved in late graft rejection mediated via the indirect route, require CD40-CD40L interactions for differentiation into effectors cells, and in this respect B cells provide high levels of co-stimulation (36). B cells may also modulate many aspects of DC function including antigen presentation and migration (34). In the present study, FTY720 markedly reduced the surface MHC class II expression on lymph node B cells. The fact that the most affected B cell population is also CD40+ may suggest that FTY720 interferes with B cell activation.

Our data confirm that FTY720 is an effective immunosuppressant in corneal allograft rejection with potential to reverse rejection even after priming. We show that FTY720 limits the early corneal infiltration with CD11c+ cells and prevents both T and B cell expansion in the DLN with subsequent impairment of late recruitment of inflammatory cells into the graft. Additionally, FTY720 appears to have a significant downregulatory effects on MHC class II expression on B cells in draining lymph nodes.

ACKNOWLEDGMENT

The authors would like to thank members of MRF, University of Aberdeen for their excellent technical assistance and Novartis Institute for BioMedical Research for synthesis of FTY720. Work was supported by the Development Trust, University of Aberdeen.

FIGURE LEGENDS

FIGURE 1. The influence of timing of FTY720 administration on rejection of mouse corneal grafts (BALB/c→C57Bl/6). Mice were treated daily with saline or with FTY720 for specified time intervals (-2 to 6d, -2 to 20d, 2 to 12d, 6 to 12d, 6 to 20d) and examined for signs of graft rejection. The data show days of rejection (A) or intervals (in days) from FTY720 withdrawal to graft rejection (B) in individual groups. Small horizontal lines represent median value. (*$p<0.001$ in comparison with control group).

FIGURE 2. Immunohistological analysis of the effect of FTY720 on initial (2.5d) and late (20d) cellular infiltration of grafted eyes. (A) Immunohistology shows a stronger cellular infiltration in host than donor tissue at 2.5d post graft. No differences were observed in CD11b, MHC Class II, or F4/80 staining at this time but a significant ($p < 0.01$) reduction in CD11c+ cells was observed in the recipient cornea in the FTY720-treated mice. Clinical appearance (B) and immunohistological analysis (C) of grafts 20d after transplantation disclose almost complete suppression of the inflammatory response and neovascularization in grafted eyes treated with FTY720 from -2d to 20d. Bars represent average number + standard deviation (SD) of cells in two optical fields at 400x magnification. (* $p<0.01$; ** $p<0.001$).

FIGURE 3. Weight of SMDLN (A) and CSLN (B) after transplantation. Mice were treated with FTY720 for indicated periods and the samples were analyzed on the last day of FTY720 administration. Pooled results of 2-3 independent experiments (a total 4-12 mice per group) are presented (mean + SD). (* $p<0.05$; ** $p<0.01$; *** $p<0.005$; **** $p<0.001$).

FIGURE 4. Apoptosis level in draining and non-draining lymph nodes after corneal transplantation. Sections of draining and non-draining lymph nodes in saline- and FTY720-treated mice 20d after transplantation were stained by the TUNEL method. The bars show the average number of TUNEL-positive cells in 1 mm^2 + SD. (The data represents 80 lymph node sections evaluated).

FIGURES

Figure 1

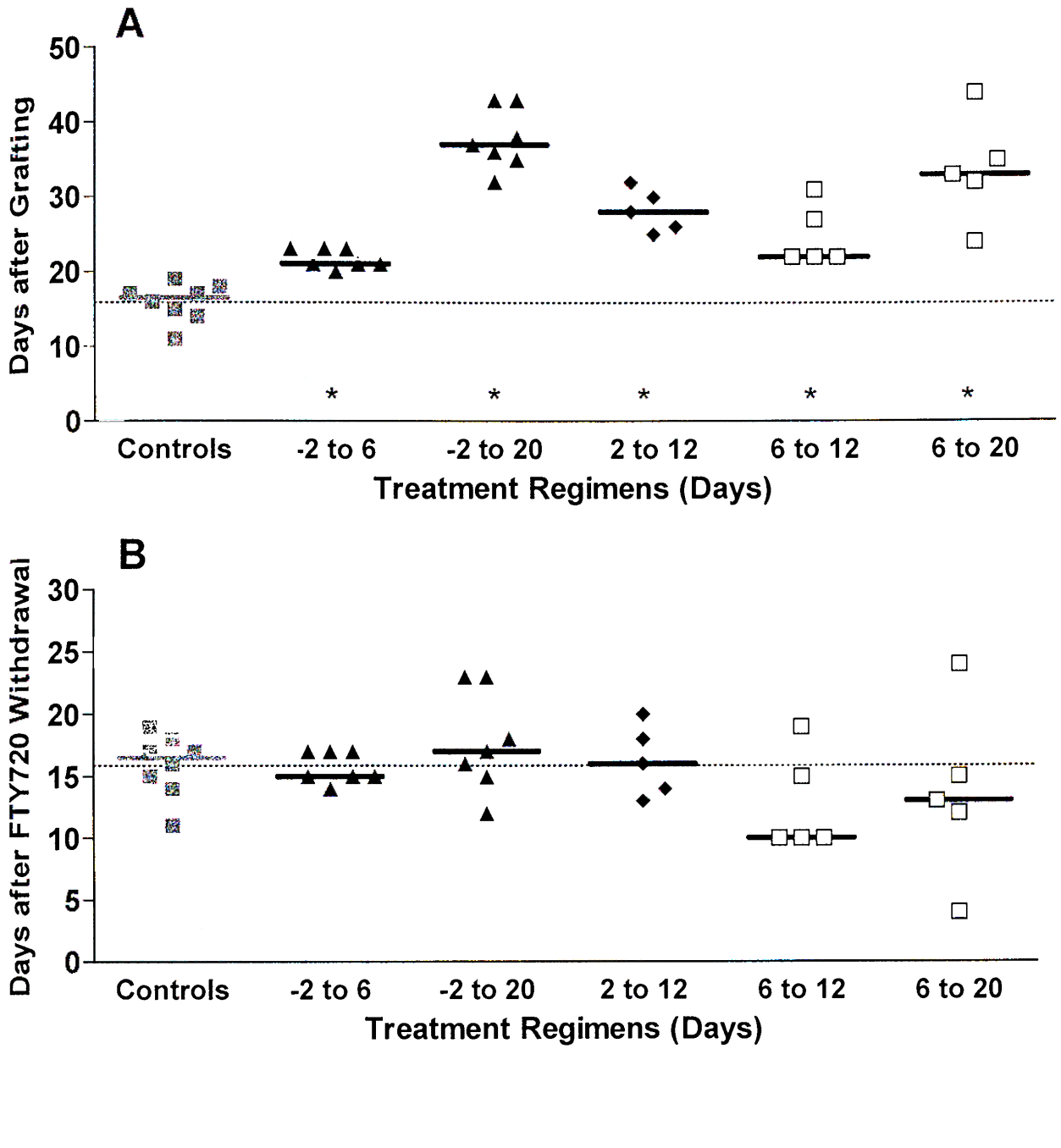


Figure 2

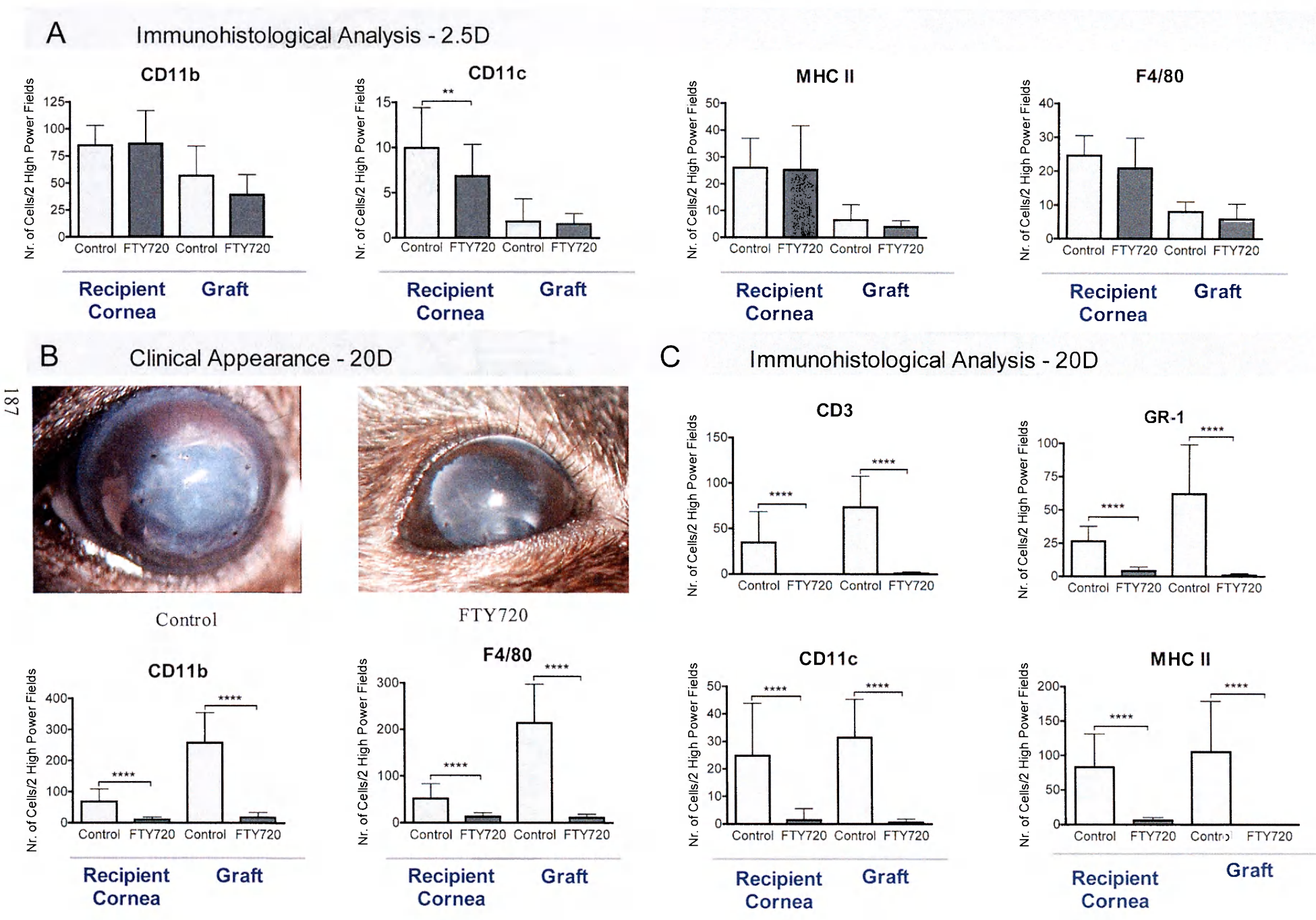

A
Immunohistological Analysis - 2.5D
CD11b
CD11c
MHC II
F4/80
Nr. of Cells/2 High Power Fields
Control FTY720 Control FTY720
Recipient Cornea
Graft
**
B
Clinical Appearance - 20D
Control
FTY720
C
Immunohistological Analysis - 20D
CD3
GR-1
CD11b
F4/80
CD11c
MHC II
****

Figure 3

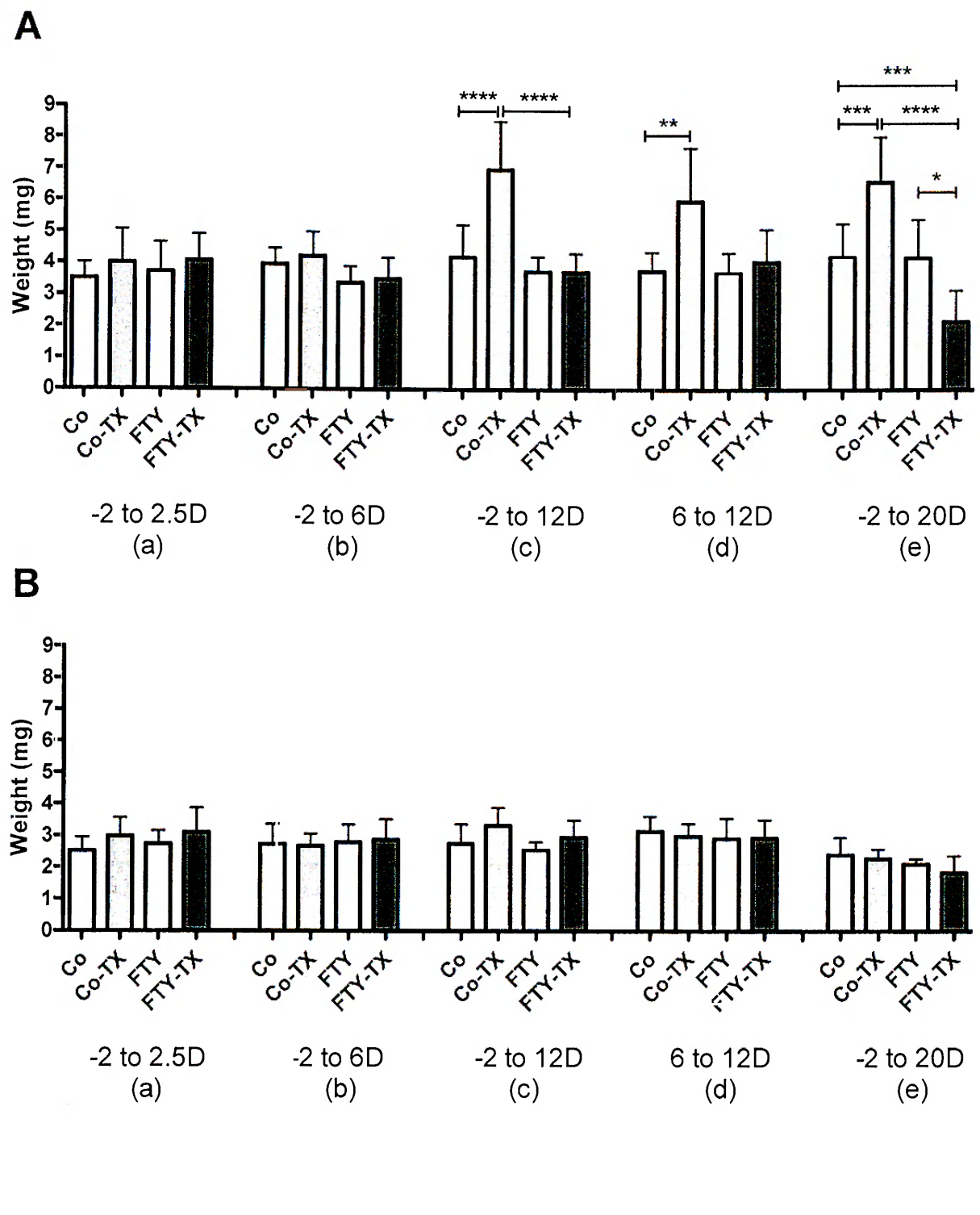

A
Weight (mg)
0
1
2
3
4
5
6
7
8
9
Co
Co-TX
FTY
FTY-TX
****
****
**
***
***
****
*
-2 to 2.5D
(a)
-2 to 6D
(b)
-2 to 12D
(c)
6 to 12D
(d)
-2 to 20D
(e)
B
Weight (mg)
-2 to 2.5D
(a)
-2 to 6D
(b)
-2 to 12D
(c)
6 to 12D
(d)
-2 to 20D
(e)

Figure 4

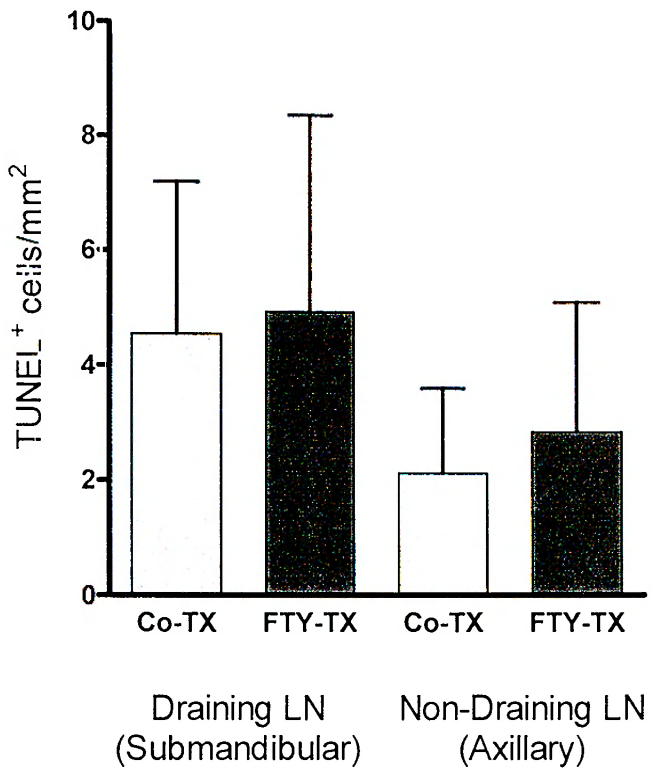

10
8
6
4
2
0
TUNEL+ cells/mm2
Co-TX
FTY-TX
Co-TX
FTY-TX
Draining LN
(Submandibular)
Non-Draining LN
(Axillary)

TABLE 1. Flow cytometry analysis of draining lymph node cell populations

Number of cells ($x10^6$) (Mean ± SD)	**Group**[a]	**-2 to 2.5d**	**-2 to 6d**	**-2 to 12d**	**6 to 12d**	**-2 to 20d**
Total	Co	4.9 ± 3.1	5.2 ± 1.3	3.9 ± 1.7	3.0 ± 1.3	5.2 ± 1.6
	Co-TX	4.3 ± 1.2	4.5 ± 1.3	7.2 ± 2.2[b]	5.6 ± 1.0[c]	7.6 ± 2.6
	FTY	3.8 ± 1.5	4.5 ± 0.9	3.3 ± 0.4	3.8 ± 0.8	3.6 ± 0.8
	FTY-TX	4.4 ± 1.3	4.4 ± 1.5	3.0 ± 1.3	4.3 ± 2.3	2.3 ± 0.4[b]
CD3+						
	Co	3.9 ± 2.6	3.6 ± 1.0	2.9 ± 1.3	2.0 ± 1.0	3.1 ± 1.2
	Co-TX	3.0 ± 0.9	3.2 ± 0.9	5.0 ± 1.4[b]	3.0 ± 0.8	3.6 ± 1.2
	FTY	2.7 ± 1.0	2.8 ± 0.7	2.3 ± 0.3	1.9 ± 0.5	2.1 ± 0.7
	FTY-TX	3.1 ± 0.8	2.7 ± 1.0	2.1 ± 0.9	2.2 ± 1.2	1.3 ± 0.2[b]
B220+						**MHC II+**
	Co	1.6 ± 0.8	2.2 ± 0.6	1.2 ± 0.5	0.7 ± 0.4	2.5 ± 0.7
	Co-TX	1.6 ± 0.5	2.1 ± 0.7	2.2 ± 0.8[b]	1.5 ± 0.2[c]	4.6 ± 2.5[b]
	FTY	1.5 ± 0.9	1.9 ± 0.3	1.2 ± 0.2	1.0 ± 0.1	1.8 ± 0.8
	FTY-TX	1.8 ± 0.7	2.0 ± 0.6	0.9 ± 0.4	1.6 ± 0.5	1.1 ± 0.3[b]
CD11c+						
	Co	0.12 ± 0.06	0.11 ± 0.03	0.08 ± 0.03	0.05 ± 0.02	0.18 ± 0.04
	Co-TX	0.11 ± 0.03	0.09 ± 0.03	0.16 ± 0.06[b]	0.11 ± 0.01[c]	0.21 ± 0.06
	FTY	0.09 ± 0.05	0.08 ± 0.01	0.07 ± 0.03	0.06 ± 0.02	0.14 ± 0.05
	FTY-TX	0.11 ± 0.04	0.08 ± 0.03	0.05 ± 0.03	0.07 ± 0.05	0.05 ± 0.01
Fluorescence intensity (Geomean ± SD)	**B220+CD40+ MHC II**					**CD40+ MHC II**
	Co	341 ± 22	914 ± 51	550 ± 48	623 ± 76	206 ± 28
	Co-TX	348 ± 50	952 ± 115	764 ± 144[c]	693 ± 114	180 ± 38
	FTY	129 ± 20[b]	385 ± 70[b]	212 ± 32[b]	251 ± 9[b]	130 ± 15[b]
	FTY-TX	145 ± 25[b]	383 ± 94[b]	225 ± 36[b]	262 ± 36[c]	109 ± 35[b]

[a] "Co" - saline treated non-transplanted controls, "Co-TX" - saline treated transplanted control, "FTY" - FTY720 treated non-transplanted mice and "FTY-TX" - FTY720 treated transplanted mice; the lymph nodes were analysed on the last day of the treatment.
[b] Statistically significant ($p < 0.005$ to 0.05) vs. control group
[c] $p = 0.057$ vs. control group

REFERENCES

1. Williams KA, Roder D, Esterman A, Muehlberg SM, Coster DJ. Factors predictive of corneal graft survival. Report from the Australian Corneal Graft Registry. Ophthalmology 1992; 99: 403.
2. Birnbaum F, Bohringer D, Sokolovska Y, Sundmacher R, Reinhard T. Immunosuppression with cyclosporine A and mycophenolate mofetil after penetrating high-risk keratoplasty: a retrospective study. Transplantation 2005; 79: 964.
3. Chiba K, Hoshino Y, Suzuki C, et al. FTY720, a novel immunosuppressant possessing unique mechanisms. I. Prolongation of skin allograft survival and synergistic effect in combination with cyclosporine in rats. Transplant Proc 1996; 28: 1056.
4. Suzuki S, Enosawa S, Kakefuda T, et al. Immunosuppressive effect of a new drug, FTY720, on lymphocyte responses in vitro and cardiac allograft survival in rats. Transpl Immunol 1996; 4: 252.
5. Wang ME, Tejpal N, Qu X, et al. Immunosuppressive effects of FTY720 alone or in combination with cyclosporine and/or sirolimus. Transplantation 1998; 65: 899.
6. Mitsusada M, Suzuki S, Kobayashi E, Enosawa S, Kakefuda T, Miyata M. Prevention of graft rejection and graft-versus-host reaction by a novel immunosuppressant, FTY720, in rat small bowel transplantation. Transpl Int 1997; 10: 343.
7. Ferguson R. FTY720 immunomodulation: optimism for improved transplant regimens. Transplant Proc 2004; 36: 549S.

8. Popovic J, Kover KL, Moore WV. The effect of immunomodulators on prevention of autoimmune diabetes is stage dependent: FTY720 prevents diabetes at three different stages in the diabetes-resistant biobreeding rat. Pediatr Diabetes 2004; 5: 3.

9. Kurose S, Ikeda E, Tokiwa M, Hikita N, Mochizuki M. Effects of FTY720, a novel immunosuppressant, on experimental autoimmune uveoretinitis in rats. Exp Eye Res 2000; 70: 7.

10. Fujino M, Funeshima N, Kitazawa Y, et al. Amelioration of experimental autoimmune encephalomyelitis in Lewis rats by FTY720 treatment. J Pharmacol Exp Ther 2003; 305: 70.

11. Matsuura M, Imayoshi T, Chiba K, Okumoto T. Effect of FTY720, a novel immunosuppressant, on adjuvant-induced arthritis in rats. Inflamm Res 2000; 49: 404.

12. Haskova Z, Usiu N, Pepose JS, Ferguson TA, Stuart PM. CD4+ T cells are critical for corneal, but not skin, allograft rejection. Transplantation 2000; 69: 483.

13. Zhang EP, Muller A, Ignatius R, Hoffmann F. Significant prolongation of orthotopic corneal-graft survival in FTY720-treated mice. Transplantation 2003; 76: 1511.

14. Sedlakova K, Muckersie E, Robertson M, Filipec M, Forrester JV. FTY720 in corneal concordant xenotransplantation. Transplantation 2005; 79: 297.

15. Mayer K, Birnbaum F, Reinhard T, et al. FTY720 prolongs clear corneal allograft survival with a differential effect on different lymphocyte populations. Br J Ophthalmol 2004; 88: 915.

16. Henning G, Ohi L, Junt T, et al. CC chemokine receptor 7-dependent and -independent pathways for lymphocyte homing: modulation by FTY720. J Exp Med 2001; 194: 1875.

17. Pabst O, Herbrand H, Willenzon S, et al. Enhanced FTY720-mediated lymphocyte homing requires G alpha i signaling and depends on beta 2 and beta 7 integrin. J Immunol 2006; 176: 1474.

18. Matloubian M, Lo CG, Cinamon G, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature 2004; 427: 355.

19. Isoyama N, Takai K, Tsuchida M, Matsumura M, Naito K. Evidence that FTY720 induces rat thymocyte apoptosis. Transpl Immunol 2006; 15: 265.

20. Vaessen LM, van Besouw NM, Mol WM, Ijzermans JN, Weimar W. FTY720 treatment of kidney transplant patients: a differential effect on B cells, naive T cells, memory T cells and NK cells. Transpl Immunol 2006; 15: 281.

21. Graler MH, Goetzl EJ. The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors. Faseb J 2004; 18: 551.

22. Nagahara Y, Ikekita M, Shinomiya T. T cell selective apoptosis by a novel immunosuppressant, FTY720, is closely regulated with Bcl-2. Br J Pharmacol 2002; 137: 953.

23. Suzuki S, Enosawa S, Kakefuda T, et al. A novel immunosuppressant, FTY720, with a unique mechanism of action, induces long-term graft acceptance in rat and dog allotransplantation. Transplantation 1996; 61: 200.

24. Sanchez T, Estrada-Hernandez T, Paik JH, et al. Phosphorylation and action of the immunomodulator FTY720 inhibits vascular endothelial cell growth factor-induced vascular permeability. J Biol Chem 2003; 278: 47281.

25. Peng X, Hassoun PM, Sammani S, et al. Protective effects of sphingosine 1-phosphate in murine endotoxin-induced inflammatory lung injury. Am J Respir Crit Care Med 2004; 169: 1245.

26. Singer, II, Tian M, Wickham LA, et al. Sphingosine-1-phosphate agonists increase macrophage homing, lymphocyte contacts, and endothelial junctional complex formation in murine lymph nodes. J Immunol 2005; 175: 7151.

27. Kuffova L, Lumsden L, Vesela V, et al. Kinetics of leukocyte and myeloid cell traffic in the murine corneal allograft response. Transplantation 2001; 72: 1292.

28. Lan YY, De Creus A, Colvin BL, et al. The sphingosine-1-phosphate receptor agonist FTY720 modulates dendritic cell trafficking in vivo. Am J Transplant 2005; 5: 2649.

29. Yagi H, Kamba R, Chiba K, et al. Immunosuppressant FTY720 inhibits thymocyte emigration. Eur J Immunol 2000; 30: 1435.

30. Chiba K, Yanagawa Y, Masubuchi Y, et al. FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. I. FTY720 selectively decreases the number of circulating mature lymphocytes by acceleration of lymphocyte homing. J Immunol 1998; 160: 5037.

31. Cyster JG. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. Annu Rev Immunol 2005; 23: 127.

32. Yamada J, Kurimoto I, Streilein JW. Role of CD4+ T cells in immunobiology of orthotopic corneal transplants in mice. Invest Ophthalmol Vis Sci 1999; 40: 2614.

33. Muller H, Hofer S, Kaneider N, et al. The immunomodulator FTY720 interferes with effector functions of human monocyte-derived dendritic cells. Eur J Immunol 2005; 35: 533.

34. Chesnut RW, Grey HM. Antigen presentation by B cells and its significance in T-B interactions. Adv Immunol 1986; 39: 51.

35. Crawford A, Macleod M, Schumacher T, Corlett L, Gray D. Primary T cell expansion and differentiation in vivo requires antigen presentation by B cells. J Immunol 2006; 176: 3498.

36. MacLeod M, Kwakkenbos MJ, Crawford A, et al. CD4 memory T cells survive and proliferate but fail to differentiate in the absence of CD40. J Exp Med 2006; 203: 897.

37. Mattila P, Hayry P, Renkonen R. Protein kinase C is crucial in signal transduction during interferon-gamma induction in endothelial cells. Transplant Proc 1990; 22: 130.

38. Hwang MW, Matsumori A, Furukawa Y, et al. FTY720, a new immunosuppressant, promotes long-term graft survival and inhibits the progression of graft coronary artery disease in a murine model of cardiac transplantation. Circulation 1999; 100: 1322.

Chapter 8

FTY720 Affects MHC Class II Expression on Lymph Node B Cells

K. Sedláková, M. Robertson, E. Muckersie, L. Duncan, M. Filipec and J. V. Forrester

Submitted for publication

FTY720 Affects MHC Class II Expression on Lymph Node B Cells

(Short communication)

Running title: FTY720 and MHC Class II Expression

K. Sedláková[1,2], M. Robertson[1], E. Muckersie[1], L. Duncan[1], M. Filipec[3,4] and J. V. Forrester[1]

(Submitted for publication)

[1] Department of Ophthalmology, IMS, University of Aberdeen, Scotland, UK

[2] Department of Ophthalmology, 1st Medical Faculty of Charles University and General Faculty Hospital, Prague 2, Czech Republic

[3] Laboratory of Ophthalmology and Eye Bank, 1st Medical Faculty and General Faculty Hospital, Charles University, Prague 2, Czech Republic

[4] Eye Clinic Lexum, Prague 4, Czech Republic

Corresponding author: J.V. Forrester, Department of Ophthalmology, IMS, University of Aberdeen, Medical School, AB25 2ZD, Scotland, UK. Tel.: +44 1224 553782, fax: +44 1224 555955, e-mail: j.forrester@abdn.ac.uk

Keywords: FTY720 / B cell / MHC class II / lymph node

Abbreviations: LPS – lipopolysaccharide; MHC – major histocompatibility complex; PKC - protein kinase C; S1P - sphingosine-1 phosphate

Abstract

FTY720 is an effective immunosuppressant in various experimental transplantation and autoimmune models. Our previous experiments demonstrated that FTY720 significantly prolonged corneal graft survival, markedly restricted the expansion of T and B cells in draining lymph nodes and reduced expression of MHC class II molecules on the surface of B cells *in vivo*. The results suggest that FTY720 may affect antigen presentation. This study examined the effects of FTY720 on MHC class II expression on B220+ and CD11c+ cells from lymph nodes and the spleen *in vitro*. B220+ cells from lymph nodes cultured in the presence of 50 nM FTY720 or from FTY720 treated mice expressed significantly reduced levels of MHC class II molecules on their surface ($p < 0.05$) in contrast to B220+ cells from the spleen or CD11C+ cells. Decreased MHC class II expression was partially restored by stimulation with lipopolysaccharide. In this study, we demonstrate that FTY720 reduces expression of MHC class II molecules on the surface of B220+ cells from lymph nodes which may represent an additional mechanism of FTY720-induced immunosuppression.

FTY720, a novel immunosuppressant, is known to prevent graft rejection in several experimental models (e.g. skin (Chiba et al., 1996), heart (Suzuki et al., 1996), or corneal alio- (Zhang et al., 2003) and xenografts (Sedlakova et al., 2005)) and is also effective in models of autoimmunity (Fujino et al., 2003) and thus potentially has wide applicability.

FTY720, a sphingosine analogue, acts in its phosphorylated form as an agonist on type 1, 3, 4 and 5 sphingosine-1 phosphate (S1P) receptors (Brinkmann et al., 2002). In its non-phosphorylated form it acts as a non-competitive inhibitor of type 1, 5 (with high potency) and 2 (with moderate potency) sphingosine receptors at nanomolar concentrations (Graler and Goetzl, 2004) inducing their prolonged internalization and partial degradation. Its immunosuppressive effect is thought to be result of sequestration of T and B cells in lymph nodes and Peyer's patches through the loss of responsiveness toward plasmatic S1P and downregulation of S1P1 receptors by FTY720 (Henning et al., 2001; Graler and Goetzl, 2004), however, its precise mode of action remains to be clarified.

We showed previously that FTY720 markedly restricts the expansion of T and B cells in draining lymph nodes that is normally triggered by transplantation and reduces the expression of MHC class II molecules on lymph node B cells *in vivo* (Sedlakova et al., 2005). This may suggest that FTY720 interferes also with antigen presentation. The study demonstrates that FTY720 reduces levels of MHC class II on the surface of B220+ cells from lymph nodes but not from the spleen and not on CD11c+ cells *in vitro*.

Material and Methods

Animals

Female C57Bl/6 mice (10- to 18-week-old) were obtained from the Biological Services Unit, University of Aberdeen and handled in accordance with the UK Animal License Act 1986 and ARVO Statement for the Use of Animals in Ophthalmic and Visual Research.

Treatment protocol and culture of lymph node cells

Three groups of mice were treated daily intraperitonealy for 48 hours: saline treated mice received sterile saline only (group 1, n = 4), saline treated mice whose cells were subsequently cultured for 24 hours with 50 nM FTY720 (group 2, n = 4) and FTY720-treated mice received 0.5 mg/kg/day of FTY720 (kindly provided by Novartis (Basel, Switzerland)) (group 3, n = 4). Single cell suspensions (n = 8 per group) were prepared from pooled cervical, brachial and inguinal lymph nodes and $1x10^6$ cells were cultured for 24 hours in 1 ml of complete medium [RPMI 1640 (Gibco™ Invitrogen Corporation, Paisley, UK)] with 5% fetal calf serum (Sigma, Poole, Dorset, UK), L-glutamine (200 mM), sodium pyruvate MEM (100 mM), 2-mercaptoethanol (50 mM), MEM nonessential amino acids solution, 5000 IU/ml of penicillin and 5 mg/ml of streptomycin (all from Gibco™ Invitrogen Corporation, Paisley, UK) in 24-well plate (Nunclon™, Δ Surface, Nalge Nunc Int., Roskilde, Denmark). Half of the samples were stimulated with 1 μg/ml LPS (Sigma, Poole, Dorset, UK). The cells were harvested with 20 mM EDTA (Sigma, Poole, Dorset, UK) and after two washes were treated for 20 minutes on ice in the dark with Fc-Bloc (rat anti-mouse CD16/32, BD Biosciences -Pharmingen, Oxford, UK in staining buffer (1% foetal calf serum plus 1% NaN3 in PBS) and then stained with

the following monoclonal antibodies in staining buffer for 30 minutes; PE-labelled anti-mouse I-A^d, APC-labelled anti-mouse CD11c, APC-Cy7-labelled anti-mouse CD45R/B220 and appropriate isotype controls (BD Biosciences -Pharmingen, Oxford, UK). After two washes, cells were fixed with CellFIX™ (BD Biosciences - Pharmingen, Oxford, UK) for a further 30 minutes, washed again and analyzed using a LSR Flow Cytometer (BD Biosciences -Pharmingen, Oxford, UK). Data were analyzed using FlowJo (4.5.2) software. Experiment was repeated twice.

Statistical analysis

Mann-Whitney test was used for statistical analysis and probability values below 0.05 were considered statistically significant.

Results and Discussion

This study examines the effects of FTY720 on MHC class II expression on B220+ and CD11c+ cells from lymph nodes and the spleen *in vitro*. Cultured lymph node B220+ cells in the presence of 50 nM FTY720 or from FTY720 treated mice expressed markedly reduced levels of MHC class II on their surface ($p < 0.05$) (Fig. 1). Interestingly, CD11c+ cells did not show any reduction in MHC class II expression similarly to B220+ cells from the spleen (data not shown). Reduced MHC class II expression on B cells may negatively affect a number of immune processes in which they are involved - antigen presentation (Chesnut and Grey, 1986), formation of aggregates with T cells (Sanders et al., 1987) and their responsiveness to T cell derived help (Bottomly et al., 1983). Recent studies of the antigen presenting function of B cells have highlighted their essential role in sustaining the immune response after the initial induction by dendritic cells (Crawford et al., 2006).

Upregulation of MHC class II expression on B cells can be induced, for instance, by the activation of protein kinase C (PKC), crosslinking of surface immunoglobulins, by IL-4 or LPS (Mond et al., 1981; Lindsten et al., 1984; Sanders et al., 1987). A few agents have been reported to downregulate MHC class II on B cells (e.g. dexamethasone, ethanol, prostaglandin E_2 or cAMP in combination with theophilline (Snyder et al., 1982; Coggeshall and Cambier, 1985; Dennis and Mond, 1986; Weiss et al., 1996)). Some like dexamethasone were shown to downregulate MHC class II also in unstimulated B cells, while others (prostaglandin E_2) were not. Sphingosine, of which FTY720 is an analogue, interferes with the activation of PKC and can downregulate interferon-γ-induced MHC class II

expression on rat microvascular endothelial cells (Mattila et al., 1990). In addition, FTY720 downregulates interferon-γ-induced expression MHC class I and class II on adherent peritoneal macrophages (Hwang et al., 1999).

The data provided by this study indicate that although FTY720 restricts MHC class II expression on lymph node B cells, additional stimulation with LPS is capable of partially restoring this expression ($p < 0.05$), although significantly below that of similarly treated controls ($p < 0.05$) (Fig. 1). While PKC is necessary for B cell receptor-induced nuclear factor-κB activation, it is not required for CD40- or LPS-induction of nuclear factor-κB (Francis et al., 1995). These results support the assumption that the FTY720-mediated effect on B cells´ MHC class II expression may be caused by interference with PKC activation. We show that another pathway involved in MHC class II regulation can to some extent operate in the presence of FTY720.

In conclusion, FTY720 reduces levels of MHC class II expression on the surface of lymph node B220+ cells, which may represent an additional mechanism of FTY720-induced immunosuppression.

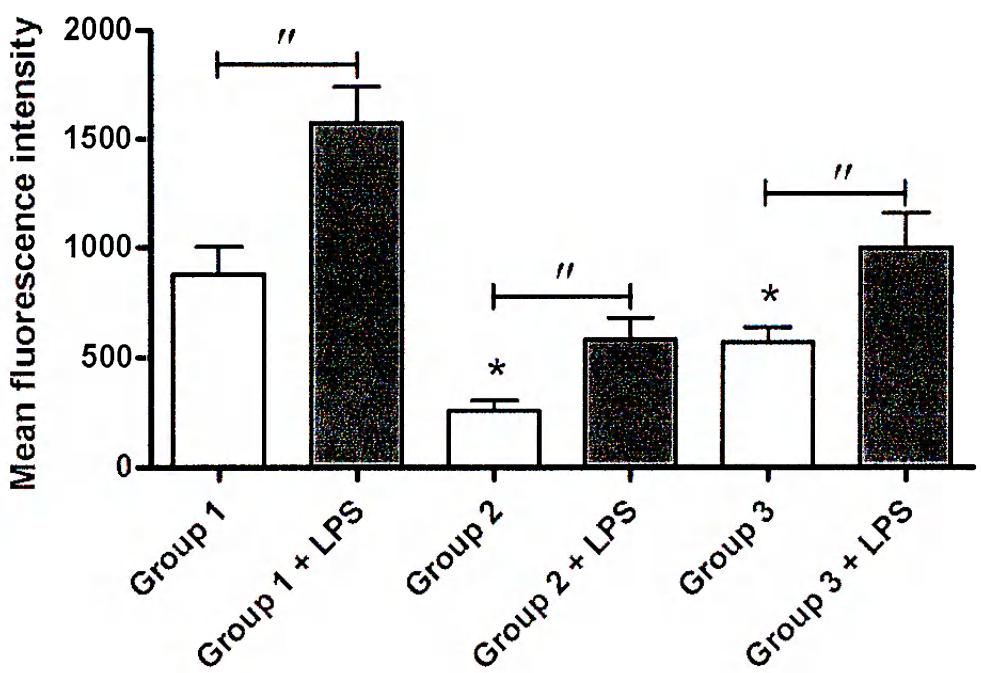
Acknowledgment

The authors would like to thank members of MRF, University of Aberdeen for their excellent technical assistance and Assoc. Prof. Vladimir Holáň for critical reading of the manuscript. Work was supported by the Development Trust, University of Aberdeen.

Figure legend

Fig. 1. MHC class II expression on B220+ cells from lymph nodes *in vitro* (a) cells from mice treated for 48 hours with saline and cultured for additional 24 hours in complete medium (group 1); (b) cells treated as group 1 with addition of 50 nM FTY720 into culture; (c) cells from mice treated for 48 hours with 0.5 mg/kg/day FTY720 and cultured as group 1 (group 3). Half of the samples was stimulated with 1 μg/ml LPS. * $p<0.05$ vs. control group 1, " $p<0.05$ vs. unstimulated cells.

Figure 1



References

Bottomly, K., Jones, B., Kaye, J., Jones, F., 3rd (1983) Subpopulations of B cells distinguished by cell surface expression of Ia antigens. Correlation of Ia and idiotype during activation by cloned Ia-restricted T cells. *J Exp Med* **158,** 265-279.

Brinkmann, V., Davis, M. D., Heise, C. E., Albert, R., Cottens, S., Hof, R., Bruns, C., Prieschl, E., Baumruker, T., Hiestand; P., Foster, C. A., Zollinger, M., Lynch, K. R. (2002) The immune modulator FTY720 targets sphingosine 1-phosphate receptors. *J Biol Chem* **277,** 21453-21457.

Coggeshall, K. M., Cambier, J. C. (1985) B cell activation. VI. Effects of exogenous diglyceride and modulators of phospholipid metabolism suggest a central role for diacylglycerol generation in transmembrane signaling by mIg. *J Immunol* **134,** 101-107.

Crawford, A., Macleod, M., Schumacher, T., Corlett, L., Gray, D. (2006) Primary T cell expansion and differentiation in vivo requires antigen presentation by B cells. *J Immunol* **176,** 3498-3506.

Dennis, G., Mond, J. (1986) Corticosteroid-induced suppression of murine B cell immune response antigens. *J Immunol* **136,** 1600-1604.

Francis, D. A., Karras, J. G., Ke, X. Y., Sen, R., Rothstein, T. L. (1995) Induction of the transcription factors NF-kappa B, AP-1 and NF-AT during B cell stimulation through the CD40 receptor. *Int Immunol* **7,** 151-161.

Fujino, M., Funeshima, N., Kitazawa, Y., Kimura, H., Amemiya, H., Suzuki, S., Li, X. K. (2003) Amelioration of experimental autoimmune encephalomyelitis in Lewis rats by FTY720 treatment. *J Pharmacol Exp Ther* **305,** 70-77.

Graler, M. H., Goetzl, E. J. (2004) The immunosuppressant FTY720 down-regulates sphingosine 1-phosphate G-protein-coupled receptors. *Faseb J* **18,** 551-553.

Henning, G., Ohi, L., Junt, T., Reiterer, P., Brinkmann, V., Nakano, H., Hohenberger, W., Lipp, M., Forster, R. (2001) CC chemokine receptor 7-dependent and -independent pathways for lymphocyte homing: modulation by FTY720. *J Exp Med* **194,** 1875-1881.

Hwang, M. W., Matsumori, A., Furukawa, Y., Ono, K., Okada, M., Iwasaki, A., Hara, M., Sasayama, S. (1999) FTY720, a new immunosuppressant, promotes long-term graft survival and inhibits the progression of graft coronary artery disease in a murine model of cardiac transplantation. *Circulation* **100,** 1322-1329.

Chesnut, R. W., Grey, H. M. (1986) Antigen presentation by B cells and its significance in T-B interactions. *Adv Immunol* **39,** 51-94.

Chiba, K., Hoshino, Y., Suzuki, C., Masubuchi, Y., Yanagawa, Y., Ohtsuki, M., Sasaki, S., Fujita, T. (1996) FTY720, a novel immunosuppressant possessing unique mechanisms. I. Prolongation of skin allograft survival and synergistic effect in combination with cyclosporine in rats. *Transplant Proc* **28,** 1056-1059.

Lindsten, T., Thompson, C. B., Finkelman, F. D., Andersson, B., Scher, I. (1984) Changes in the expression of B cell surface markers on complement receptor-positive and complement receptor-negative B cells induced by phorbol myristate acetate. *J Immunol* **132,** 235-239.

Mattila, P., Hayry, P., Renkonen, R. (1990) Protein kinase C is crucial in signal transduction during interferon-gamma induction in endothelial cells. *Transplant Proc* **22,** 130.

Mond, J. J., Seghal, E., Kung, J., Finkelman, F. D. (1981) Increased expression of I-region-associated antigen (Ia) on B cells after cross-linking of surface immunoglobulin. *J Immunol* **127,** 881-888.

Sanders, V. M., Fernandez-Botran, R., Uhr, J. W., Vitetta, E. S. (1987) Interleukin 4 enhances the ability of antigen-specific B cells to form conjugates with T cells. *J Immunol* **139,** 2349-2354.

Sedlakova, K., Muckersie, E., Robertson, M., Filipec, M., Forrester, J. V. (2005) FTY720 in corneal concordant xenotransplantation. *Transplantation* **79,** 297-303.

Snyder, D. S., Beller, D. I., Unanue, E. R. (1982) Prostaglandins modulate macrophage Ia expression. *Nature* **299,** 163-165.

Suzuki, S., Enosawa, S., Kakefuda, T., Li, X. K., Mitsusada, M., Takahara, S., Amemiya, H. (1996) Immunosuppressive effect of a new drug, FTY720, on

lymphocyte responses in vitro and cardiac allograft survival in rats. *Transpl Immunol* **4,** 252-255.

Weiss, P. A., Collier, S. D., Pruett, S. B. (1996) Role of glucocorticoids in ethanol-induced decreases in expression of MHC class II molecules on B cells and selective decreases in spleen cell number. *Toxicol Appl Pharmacol* **139,** 153-162.

Zhang, E. P., Muller, A., Ignatius, R., Hoffmann, F. (2003) Significant prolongation of orthotopic corneal-graft survival in FTY720-treated mice. *Transplantation* **76,** 1511-1513.

Chapter 9

IL-4 and vIL-10 adenovirus-mediated *ex vivo* gene transfer in rat corneal allograft transplantation

Sedláková K, Dannowski H, Filipec M, Ritter T and Pleyer U

In preparation

IL-4 and vIL-10 adenovirus-mediated e*x vivo* gene transfer in rat corneal allograft transplantation

Running title: Gene transfer in corneal allograft transplantation

Sedláková K[1,2], Dannowski H[2], Filipec M[1,3], Ritter T[4] and Pleyer U[2]

[1] Department of Ophthalmology, 1st Medical Faculty of Charles University and General Faculty Hospital, Prague, Czech Republic

[2] Department of Ophthalmology, Charité, Medical Faculty of Humboldt University, Berlin, Germany

[3] Laboratory of Ophthalmology and Eye Bank, 1st Medical Faculty of Charles University, Prague, Czech Republic

[4] Institute of Medical Immunology, Charité, Berlin, Germany

Keywords: Corneal transplantation – gene therapy – IL-4 – vIL-10

Address correspondence to: Dr. Klara Sedlakova, Department of Ophthalmology, 1st Medical Faculty of Charles University and General Faculty Hospital, Prague, Czech Republic, Tel: (+420) 224963030, Fax: (+420) 224918650, ksed@centrum.cz

ABSTRACT

Background. The gene transfer of immunomodulatory cytokines appears to be a promising approach in corneal transplantation. IL-4 and EBV-encoded IL-10 homologue (vIL-10) have prominent anti-inflammatory potential and are able to modulate the immune responses away from T_H1 towards T_H2. We wanted to determine the ability of *ex vivo* adenovirus-mediated gene transfer of these cytokines (alone or in combination) to influence corneal graft rejection in the MHC I/II mismatched rat model. **Methods.** Corneal grafts from Wistar-Furth rats were transduced *ex vivo* with: $2x10^8$ pfu/ml Ad*β-galactosidase* (β-gal; n=6), $4x10^8$ pfu/ml Adβ-gal (β-gal high; n=6), $2x10^8$ pfu/ml AdrIL-4 (IL-4; n=5), $4x10^8$ pfu/ml AdrIL-4 (IL-4 high; n=4), $1x10^8$ pfu of AdvIL-10 (vIL-10 low; n=6), $1x10^8$ pfu/ml AdrIL-4 plus $1x10^8$ pfu/ml AdvIL-10 (IL-4+vIL-10 low; n=6), and $2x10^8$ pfu/ml AdrIL-4 plus $2x10^8$ pfu/ml AdvIL-10 (IL-4+vIL-10; n=8). Nontransduced grafts (n=7) served as additional control. **Results**. Transduction protocols containing AdrIL-4 attenuated the iris vessels reaction preceding rejection in the control transplanted rats, while at the same time increased corneal graft opacity. Ex vivo transduction of corneal grafts with various treatment regimens containing AdrIL-4, AdvIL-10 or their combination was not able to prolong corneal allograft survival and co-transduction of AdrIL-4 with AdvIL-10 low adversely affected graft survival in comparison with AdrIL-4 transduction alone ($p < 0.05$). **Conclusions.** IL-4 *ex vivo* gene transfer significantly limited the post-transplant iris vessels reaction however alone or in combination was not able to significantly delay graft rejection. Signs of immune modulation that have been observed warrant further studies to clarify their role in allograft rejection.

INTRODUCTION

The cornea is one of the most frequently transplanted tissues with the overall high success of one-year acceptance. However, there remain a number of situations in which the prognosis remains poor including neovascularization, inflammation or a previous history of corneal graft rejection. In such situations, the four-year survival rate may be lower than in the case of some vascularized grafts [1,2].

Gene therapy appears to be a promising approach in many fields, including transplantation. Genes of interest (i.e. immunomodulatory) can be applied systemically or locally. Systemic administration enables us to target simultaneously various systems but it may also induce systemic immunosuppression. Local *ex vivo* genetic manipulation may suppress the ability of the graft to induce rejection, to increase its protective mechanisms and to inhibit the anti-graft immune responses. Since corneas can be easily cultured up to four weeks *in vitro*, *ex vivo* genetic manipulation appears to be a promising approach.

Corneal graft rejection is mediated mainly by donor-specific CD4+ T cells [3]. CD4+ T cells may differentiate into subsets of effector cells producing distinct sets of cytokines, and an individual T cell can produce their various mixtures. From the cytokine profile detected in the aqueous humor and within corneal grafts undergoing rejection, it can be concluded that the T_H1 response predominates in corneal graft rejection [4-6]. T_H2 type cytokines have the capacity to redirect the

recipient's immune response towards a T_H2 direction and this modification may have a positive effect on the corneal graft outcome.

IL-4 plays a major role in the promotion of the differentiation of naïve CD4+ T cells into T_H2 type cells and, once they are differentiated, IL-4 functions as their autocrine growth factor. In addition, IL-4 antagonizes the macrophage-activating effects of IFN-γ and suppresses macrophage-dependent reactions. After *ex vivo* transduction, local intraocular production of this immunomodulatory cytokine did not prolong graft survival [4-6] but increased intragraft eosinophil infiltration [6].

IL-10, produced by T_H2 cells (as well as by other cells), inhibits the activation of T_H1 cells, of macrophages and of dendritic cells and it also terminates cell-mediated immune reactions. The EBV-encoded IL-10 homologue (vIL-10) has a prominent anti-inflammatory potential and has a significant homology to human and murine IL-10 [7,8]. However, it lacks certain T cell stimulatory activities of IL-10 [9,10]. *Ex vivo* administration of AdvIL-10 significantly prolonged graft survival in sheep [11] but not in rats, where only a systemic transfer of AdvIL-10 prior transplantation prolonged corneal allograft survival [12].

This study investigated the efficacy of the combined adenovirus-mediated IL-4 and vIL-10 ex vivo gene transfer in a rat model of orthotopic corneal transplantation.

MATERIALS AND METHODS

Animals

The inbred strain of female Lewis rats (185 – 214 grams) were used as recipients of female Wistar-Furth rat corneal grafts. Animals were obtained from the M&B, Ry, Denmark and treated in accordance with the regulations of the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research and according the German law on the protection of animals and permission by the local authorities (Reg. G. 0474/95).

Ex vivo transduction of donor corneas

Corneal graft explants from Wistar-Furth rats were transduced *ex vivo* for three hours, washed, and after three hours transplanted into Lewis recipients. Different groups were transduced with various titres of vectors: $2x10^8$ pfu/ml Ad*β-galactosidase* (Dr. J.K. Kolls, New Orleans, LO, USA) (β-gal; n = 6), $4x10^8$ pfu/ml Adβ-gal (β-gal high; n = 6), $2x10^8$ pfu/ml AdrIL-4 (PD Dr. T. Ritter, Berlin) (IL-4; n = 5), $4x10^8$ pfu/ml AdrIL-4 (IL-4 high; n = 4), $1x10^8$ pfu of AdvIL-10 (PD Dr. T. Ritter, Berlin) (vIL-10 low; n = 6), $1x10^8$ pfu/ml AdrIL-4 plus $1x10^8$ pfu/ml vIL-10 (IL-4+vIL-10 low; n = 6), and $2x10^8$ pfu/ml AdrIL-4 plus $2x10^8$ pfu/ml vIL-10 (IL-4+vIL-10; n = 8). As controls, transplanted, non-transduced rats were used (n = 8) that were otherwise handled the same way, i.e. in 500 μl Dulbecco's Modified Eagle's Medium (GIBCO/BRL, Gaithersburg, MD) at 37°C in a 5% CO_2 humidified atmosphere.

Corneal transplantation

Orthotopic corneal transplantation was performed according to our standard protocol with some modifications. Briefly, recipients were anesthetized intraperitonealy with a mixture of Ketamin 500 mg Curamed (ketamin hydrochloride 50 mg/ml, CuraMED Pharma GmbH, Karlsruhe, Germany) and Rompun 2% (xylazin hydrochloride, Bayer Vital GmbH, Leverkusen, Germany) after being shortly anesthetized by inhalation of Forene (isofluran, Abbott GmbH, Wiesbaden, Germany). A mixture of Neosynephrine + Mydrum 1+3 gtt. oph. (phenylephrini hydrochloridum 25 mg/1ml + tropicamidum 3,75 mg/ml, Campus Virchow-Klinikum Apotheke, Germany) and Cyclopentolat 1% (cyclopentolat hydrochloride, Dr. Thilo & Co. GmbH, Freiburg, Germany) was used to induce mydriasis. Corneal grafts (∅ 3.5 mm) were fixed in place by running 11-0 black monofilament nylon suture (Alcon SURGICAL, USA) that was left in place for the duration of the experiment. At the end of procedure, the antibiotic ointment (Floxal, Dr. Man Pharma, Berlin, Germany) was applied once. The sutures were left in place for the duration of experiment. Healon 10 mg/ml (sodium hyaluronate 10 mg/ml, Pharmacia & Upjohn, Erlangen, Germany) was used in the course of surgery to protect the corneal endothelium. The “day of the rejection” was set as the day when the opacity of cornea increased to the extent that only pupil margin remained distinguishable (opacity >2.5).

In order to evaluate the extent of corneal neovascularization, we used a grading scheme ranging from 0 to 2. Grade 0 was assigned when no neovascularization was present, grade 0.5 and 1 when new vessels reached half the distance between the limbus and the graft margin or the graft margin respectively. "The activity of neovascularization" was graded as follows: grade 0.4 for vessels with uneven "obliterated" lumen; grade 0.6 for narrow and 0.8 for dilated vessels and grade 1.0 for greatly dilated vessels. The reaction of the iris vessels was evaluated according to the following grading score: grade 0.4, calibre of the vessels smaller than in grade 0.5; grade 0.5 calibre of the vessels in normal healthy, non-transplanted rat; grade 0.8, highly dilated iris vessels; grades 0.6 and 0.7, calibre of the vessels equally distributed between grades 0.5 and 0.8.

Statistical analysis

The Kaplan-Meier test was used to determine the statistical significance of the *ex vivo* gene transfer on the corneal graft survival and Kruskal-Wallis test to determine statistical significance of their effect on graft opacity on day 7. Significance of the effect on the iris vessel reaction was verified by Mann Whitney test. Probability values below 0.05 were considered statistically significant.

RESULTS

The effect of *ex vivo* transduction of IL-4 or vIL-10 (alone or in combination) on corneal allograft survival was examined and compared with controls (non-transduced or *ex vivo* transduced with control vector Adβ-gal). After initial postoperative corneal opacification, non-transduced, β-gal and vIL-10 transduced grafts remained clear until the onset of rejection, while grafts of groups treated with AdrIL-4 stayed opaque with an average opacity score 0.6 (AdrIL-4) and 2.0 (AdrIL-4 high) (Fig. 1A). Co-transduction with vIL-10 resulted in further increase in corneal opacity (opacity score 1.2 and 0.95) and increased activity of neovascularization (* $p < 0.01$) (Fig. 1B).

The period after corneal transplantation was associated with an increased reaction of iris vessels (grade 0.55 - 0.65) which was limited to the first few days after transplantation. After that, it was maintained on the level of 0.50 - 0.55 (Fig. 2). Transduction with higher titre of Adβ-gal (Adβ-gal high), similarly to the combination of AdrIL-4 and AdvIL-10, induced a more pronounced iris vessels reaction when compared with controls ($p = 0.0659$ and $p = 0.04$ respectively). On the other hand, transduction of corneas with AdrIL-4 ($2x10^8$ pfu/ml), alone or in combination with AdvIL-10 low, significantly reduced the iris vessels reaction as observed on day 7 after transplantation ($p < 0.005$) (Fig. 2; empty boxes).

Several days before the onset of increased corneal opacity (and rejection), the iris vessels reaction appeared again in control-transplanted rats. A similar reaction was visible in rats transplanted with corneas transduced with Adβ-gal or

AdvIL-10 low. This response was, however, significantly suppressed in all groups transduced with AdrIL-4 (administered alone as well as in combination with both concentrations of AdvIL-10) (Fig. 2; shaded boxes).

Non-transduced recipients (the control group) rejected their allografts at a median of 14 days (Fig. 3). The cytokine transduction, despite its suppressive effect on the anterior chamber reaction, showed a minimal effect on graft survival. The combination of AdrIL-4 and AdvIL-10 slightly prolonged the median survival time of grafts (MST) to 18 days but the effect was not statistically significant. Interestingly, in the post-transplantation period, two grafts from this group showed increased corneal opacity up to day 18 (1 and 2 respectively), then cleared up and remained clear until the end of observation (day 33). The only remnants of the overcome rejection episode were small keratic precipitates on the endothelium. This was not observed in any other experiment we performed.

In the pilot experiment, there was a tendency to negative correlation between the levels of vIL-10 produced during the 24-hour pre-transplant storage *in vitro* (AdvIL-10, $2x10^8$ pfu/ml) and the final graft survival. Therefore, we transduced six corneas with lower concentration of AdvIL-10 (AdvIL-10 low; $1x10^8$ pfu/ml) but this approach did not delay the onset of rejection but in fact slightly decreased graft survival (MST = 12 days). Moreover, the co-transduction with AdrIL-4 significantly reduced graft survival ($p < 0.05$) in comparison with controls or AdrIL-4 transduction alone.

DISCUSSION

In previous experiments on rat, the adenovirus-mediated *ex vivo* gene transfer of IL-4, similarly to vIL-10 [12], was not successful in the prolongation of corneal allograft survival [4]. The study investigated the outcome of rat corneal allografts after the combined *ex vivo* adenoviral transfer of IL-4 and vIL-10.

Transduction with AdrIL- 4 (alone or in combination with vIL-10) led to visible attenuation of the iris vessels reaction shortly after transplantation as well as during rejection in contrast to control mice or mice with grafts transduced with Adβ-gal or AdvIL-10 alone. This probably reflects the immunosuppressive effect of IL-4 on the inflammatory reaction in the anterior chamber.

Ex vivo corneal transduction with AdrIL-4 did not delay corneal graft rejection and, in addition, its application is dose dependently associated with increased corneal opacity. Presumably, this may result from eosinophil infiltration [6] induced by eotaxin produced by corneal fibroblasts under the influence of IL-4 [13].

Ex vivo transduction of corneal grafts with vIL-10 did not prolong the survival of corneal grafts. In some reports, AdIL-10 was described as being able to decrease the number of eosinophils and neutrophils in the bronchoalveolar lavage fluid in the mouse model of bronchial asthma [5]. However, other authors observed opposite effects [14,15]. In our model, the combined treatment of IL-4 and vIL-10 was associated with more pronounced corneal opacity and increased activity of neovascularization; the combination of IL-4 with low titre of vIL-10 shortens graft survival.

In conclusion, our attempt to redirect the local immune response towards a T_H2 type through the overexpression of IL-4 and vIL-10 after the *ex vivo* gene transfer did not prove sufficient to delay corneal allograft rejection. However, the signs of immune modulation that were observed warrant further research.

Figures legend

Fig. 1. The effect of *ex vivo* corneal transduction on (a) corneal allografts opacity and (b) activity of corneal neovascularization 7 days after transplantation. Data are expressed as mean + standard deviation (SD). (** $p < 0.01$ vs. control grafts)

Fig. 2. The reaction of iris vessels after transplantation and the effect of different treatments on day 7 and day before rejection. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ vs. results on day 7)

Fig. 3. The effect of *ex vivo* corneal transduction on rat corneal allografts survival. Grafts were transduced for three hours *ex vivo* and after additional three hours transplanted. The horizontal line represents the median survival time and ≈ represents a sign for non-rejected grafts. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$)

Figures

Figure 1

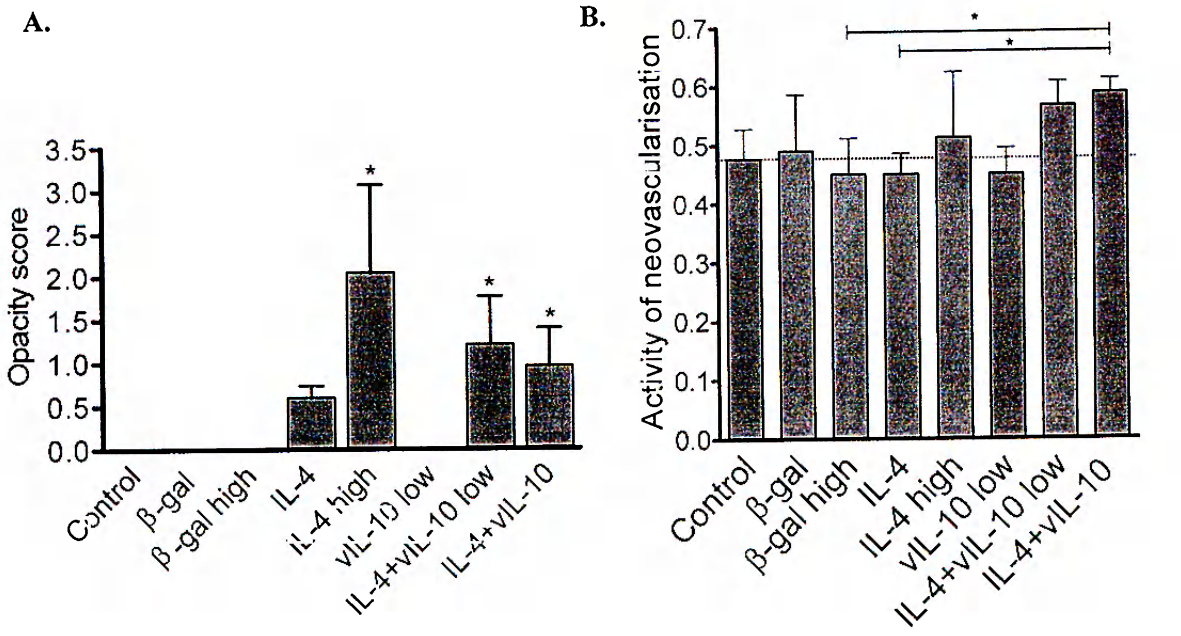


Figure 2

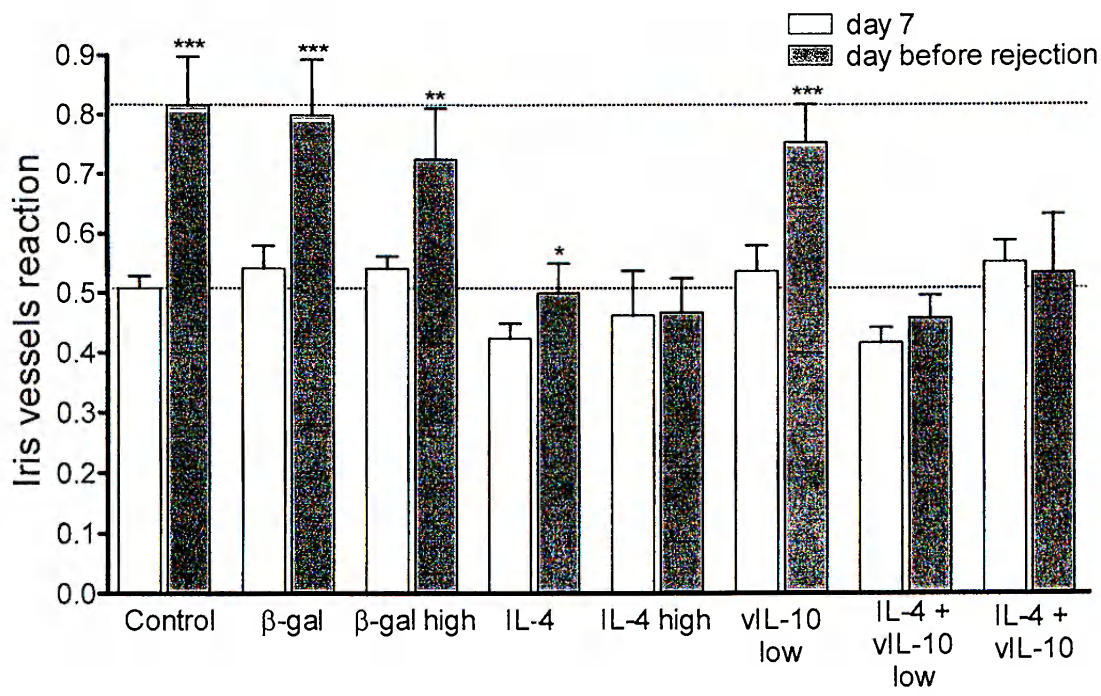
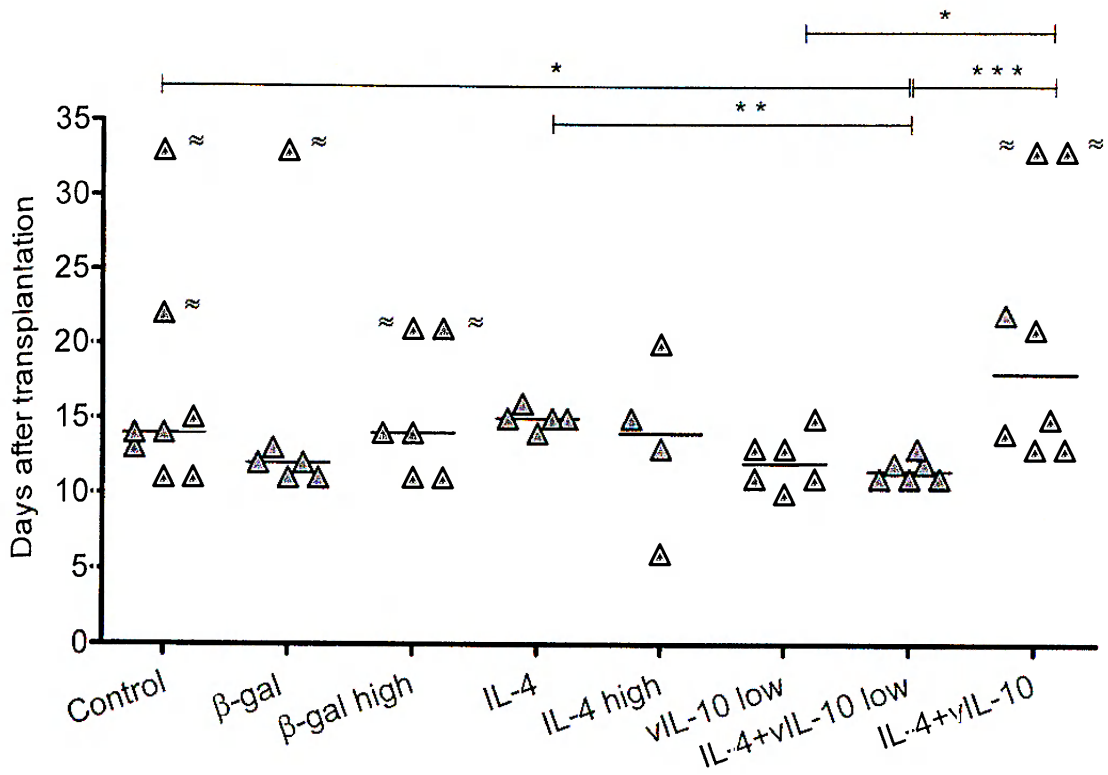

day 7
day before rejection
Iris vessels reaction
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
***
***
**
*
***
Control
β-gal
β-gal high
IL-4
IL-4 high
vIL-10 low
IL-4 + vIL-10 low
IL-4 + vIL-10

Figure 3



	Sine	β-gal	β-gal high	IL-4	IL-4 high	IL-10 low	IL-4+IL-10 low	IL-4+IL-10
Median survival time	14	12	14	15	14	12	11,5	18

REFERENCES

1 Reinhard T, Bohringer D, Enczmann J, Kogler G, Mayweg S, Wernet P, Sundmacher R: HLA class I and II matching improves prognosis in penetrating normal-risk keratoplasty. Dev Ophthalmol 2003;36:42-49.

2 Williams KA, Roder D, Esterman A, Muehlberg SM, Coster DJ: Factors predictive of corneal graft survival. Report from the Australian Corneal Graft Registry. Ophthalmology 1992;99:403-414.

3 Haskova Z, Usiu N, Pepose JS, Ferguson TA, Stuart PM: CD4+ T cells are critical for corneal, but not skin, allograft rejection. Transplantation 2000;69:483-487.

4 Pleyer U, Bertelmann E, Rieck P, Hartmann C, Volk HD, Ritter T: Survival of corneal allografts following adenovirus-mediated gene transfer of interleukin-4. Graefes Arch Clin Exp Ophthalmol 2000;238:531-536.

5 Chi-Ling Fu Y-HC, Lee-Young Chau, Bor-Luen Chiang: Effects of adenovirus-expressing IL-10 in alleviating airway inflammation in asthma. The Journal of Gene Medicine 2006;8:1393-1399.

6 Klebe S, Coster DJ, Sykes PJ, Swinburne S, Hallsworth P, Scheerlinck J-PY, Krishnan R, Williams KA: Prolongation of Sheep Corneal Allograft Survival by Transfer of the Gene Encoding Ovine IL-12-p40 but Not IL-4 to Donor Corneal Endothelium. J Immunol 2005;175:2219-2226.

7 Vieira P, de Waal-Malefyt R, Dang M, Johnson K, Kastelein R, Fiorention D, de Vries J, Roncarolo M, Mosmann T, Moore K: Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: Homology to Epstein-Barr virus open reading frame BCRFI. PNAS 1991;88:1172-1176.

8 Moore K, Vieira P, Fiorentino D, Trounstine M, Khan T, Mosmann T: Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRFI. Science 1990;248:1230-1234.

9 Ding Y, Qin L, Kotenko SV, Pestka S, Bromberg JS: A single amino acid determines the immunostimulatory activity of interleukin 10. J Exp Med 2000;191:213-224.

10 Takayama T, Tahara H, Thomson A: Differential effects of myeloid dendritic cells retrovirally transduced to express mammalian or viral interleukin-10 on cytotoxic T lymphocyte and natural killer cell functions and resistance to tumor growth. Transplantation 2001;71:1334-1340.

11 Klebe S, Sykes P, Coster D, Krishnan R, Williams K: Prolongation of sheep corneal allograft survival by ex vivo transfer of the gene encoding interleukin-10. Transplantation 2001;71:1214-1220.

12 Gong N, Pleyer U, Volk HD, Ritter T: Effects of local and systemic viral interleukin-10 gene transfer on corneal allograft survival. Gene Ther 2006;14:484-490.

13 Fukuda K: [Role of corneal fibroblasts in the pathogenesis of ocular allergic diseases]. Nippon Ganka Gakkai Zasshi 2005;109:717-726.

14 Chen D, Ding Y, Zhang N, Schroppel B, Fu S, Zang W, Zhang H, Hancock WW, Bromberg JS: Viral IL-10 gene transfer inhibits the expression of multiple chemokine and chemokine receptor genes induced by inflammatory or adaptive immune stimuli. Am J Transplant 2003;3:1538-1549.

15 Fukushima A, Sumi T, Fukuda K, Kumagai N, Nishida T, Yagita H, Ueno H: Interleukin 10 and transforming growth factor {beta} contribute to the development

of experimentally induced allergic conjunctivitis in mice during the effector phase. Br J Ophthalmol 2006;90:1535-1541.

Chapter 10

Summary of the main experimental results

1. The effect of the suturing technique on corneal xenograft survival

The use of maximally trimmed suture endings limits the degree of non-specific irritation and significantly delays the onset of rejection (60% prolongation of the mean survival time). It also decreases the induction of neovascularization.

The treatment with CsA or mAb anti-Thy-1.2 has immunosuppressive effects on corneal allograft survival comparable to the inhibition of iNOS. This observation supports our previous results demonstrating that the activation of iNOS is a T cell-dependent process.

In contrast with groups of corneal xenograft recipients with long sutures where all tested immunosuppressants (CsA, mAb anti-Thy-1.2 and AMT (a specific inhibitor of iNOS)) significantly prolong raft survival, none of these immunosuppressants shows a similar efficacy in groups of recipients with short sutures.

In summary, a simple alteration in the suturing technique significantly affects corneal concordant xenograft survival and, more importantly, affects the efficacy of immunosuppressive therapy.

2. FTY720 in corneal concordant xenotransplantation

FTY720, even in monotherapy, substantially delays the inflammatory response associated with corneal xenograft transplantation in a dose dependent manner and prevents the necrosis at the graft margin and sloughing of the xenograft, possibly enabling graft infiltration at the late time point.

Retention of the xenograft enables the coverage of the donor rat stroma by epithelium of mouse origin. Interestingly, this occurs within 9 days before the graft is clinically rejected. By the fourth week, the coverage is usually complete. At that time, immunohistological studies identify only a small number of mouse-derived epithelial cells migrating across the rat graft in control animals, indicating that reepithelialization was initiated, but is ultimately abortive.

Treatment with FTY720 induces a profound reduction in T and B cell expansion and the expression of B cell activation markers (MHC class II and CD86) with macroscopically visible prevention of the normal increase in the size of the draining lymph nodes after transplantation and massively reduces cell numbers.

3. FTY720 in corneal allograft transplantation

Monotherapy with FTY720 at a dosage as low as 0.5 mg/kg/d prevents corneal graft rejection. However, the drug is only effective if administered continuously. Clinically, corneas in treated mice are clear at the time of drug withdrawal with only minimal neovascularization on day 20 after transplantation.

Treatment with FTY720 significantly limits the early infiltration of the grafted eye with CD11c+ cells but has only a little effect on other cell populations. However, 20 days after transplantation, FTY720 considerably reduces all immune cell populations, particularly CD3+ T cells (up to 100 % reduction). These data suggest that FTY720 may selectively limit the early CD11c+ (presumed) DC recruitment to the graft, with probable downstream effects on T and B cell activation in the DLN, and impairment of the allospecific T cell and effector macrophage responses.

FTY720 markedly restricts the expansion of T and B cells in DLN that is normally triggered by transplantation. The failure of DC to traffic through the graft and transport sufficient levels of alloantigen to the DLN may contribute to reduced T and B cell activation and proliferation in the DLN but other mechanisms may be involved, since postponing the treatment with FTY720 to 6 days after transplantation produces a similar effect.

FTY720 markedly reduces the surface MHC class II expression on lymph node B cells. The fact that the most affected B cell population is also CD40+ may indicate that FTY720 interferes with B cell activation.

4. FTY720 affects MHC class II expression on lymph node B cells

Lymph node B220+ cells cultured in the presence of 50 nM FTY720 or from FTY720 treated mice (0.5 mg/kg intraperitonealy for 48 hours) express markedly reduced levels of MHC class II on their surface. Interestingly, CD11c+ cells do not show any reduction in MHC class II expression similarly to B220+ cells from the spleen.

Although FTY720 restricts MHC class II expression on lymph node B cells, additional stimulation with LPS can partially restore it. These results provide support to the assumption that the FTY720-mediated effect on B cells´ MHC class II expression may potentially be caused by interference with PKC activation.

5. IL-4 and vIL-10 adenovirus-mediated *ex vivo* gene transfer in rat corneal allograft transplantation

Transduction with AdrIL- 4 (alone or in combination with vIL-10) leads to visible attenuation of the iris vessels reaction shortly after transplantation as well as during rejection in contrast to control mice or mice with grafts transduced with Adβ-gal or AdvIL-10 alone. This probably reflects an immunosuppressive effect of IL-4 on the inflammatory reaction in the anterior chamber.

The *ex vivo* corneal transduction with AdrIL-4 does not delay corneal graft rejection and, in addition, its application is dose dependently associated with increased corneal opacity. Presumably, this may result from eosinophil

infiltration induced by eotaxin produced by corneal fibroblasts under the influence of IL-4.

Combined treatment of IL-4 and vIL-10 is associated with more pronounced corneal opacity, increased activity of neovascularization and the combination of IL-4 with low titre of vIL-10 shortens graft survival.

Chapter 11

General conclusions

1. The study of the effect of the suturing technique on corneal concordant xenograft survival provides these findings:

- The suturing technique significantly affects the outcome of corneal concordant xenograft transplantation.
- NO is involved in corneal xenograft rejection, but it not the only mechanism of rejection.
- Importantly, the suturing technique influences the effectiveness of immunosuppressive regimens. This fact must be taken into consideration when evaluating the efficacy of immunosuppressive drugs.
- Our data may therefore suggest that a substantial part of the immunosuppressive effect of selected immunosuppressants subsists in their limiting the contribution of antigen-nonspecific inflammation. Consequently, the dominant effector mechanisms responsible for xenograft rejection are not effectively compromised by this treatment.

2. The study of the efficacy and the mode of action of novel immunosuppressive FTY720 in corneal concordant xenotransplantation (rat-to-mouse) demonstrate:

- FTY720 in monotherapy significantly delays the rejection of rat-to-mouse xenogenic concordant corneal transplants in contrast with other xenogenic transplantation models.
- The severely inflamed immunologically-rejected xenograft does not support host-derived reepithelialization of the donor xenograft but treatment with FTY720 sufficiently suppresses the severity of the inflammatory response and permits the donor graft epithelial coverage by host cells and/or that FTY720 also promotes epithelial growth and migration. This is probably the first step towards permanent engraftment.
- Treatment with FTY720 induces a profound reduction in T and B cell expansion and the expression of B cell activation markers in draining lymph nodes after transplantation. Since corneal xenograft rejection is mediated not by natural antibodies or CD8+ T cells directly, but rather by CD4+ T cells, the data from these experiments imply that FTY720 mediated its effect via CD4+ T cells.
- In summary, the study shows that FTY720, used in isolation, is a potent immunosuppressant in the control of xenogenic corneal graft rejection in the rat-to-mouse model.

3. FTY720 was tested also in the corneal allotransplantation model in mouse and our results demonstrate:

- FTY720 is an effective immunosuppressant in corneal allograft rejection with a potential to reverse rejection even after priming.

- FTY720 limits the early corneal infiltration with CD11c+ cells and prevents both T and B cell expansion in the DLN with subsequent impairment of late recruitment of inflammatory cells into the graft. Additionally, FTY720 appears to have a significant downregulatory effects on MHC class II expression on B cells in draining lymph nodes.

4. The effect of FTY720 on MHC class II expression of lymph node cells and splenocytes was tested *in vivo* and *in vitro* and the findings show:

- A significant reduction of MHC class II expression on lymph node B cells can be induced by FTY720 *in vivo* as well as *in vitro*. However, this effect only relates to lymph node B cells, since CD11c+ or B220+ cells from the spleen are not affected.
- Although FTY720 restricts MHC class II expression on lymph node B cells, additional stimulation with LPS is capable of partially restoring this expression. Therefore, another pathway involved in MHC class II regulation can, to some extent, operate in the presence of FTY720.
- In conclusion, FTY720 reduces levels of MHC class II expression on the surface of lymph node B220+ cells. This may represent an additional mechanism of FTY720-induced immunosuppression.

5. The *ex vivo* IL-4 and vIL-10 adenovirus-mediated gene transfer in rat corneal allograft transplantation suggests:

- The redirection of the local immune response towards T_H2-type does not suffice to delay corneal allograft rejection. Nevertheless, the signs of immune modulations warrant further studies.

Conclusion

Corneal graft rejection represents a serious problem in a number of situations and understanding the immune mechanisms involved in the process is a fascinating task for us. Despite the progress already made, many of the mechanisms are still unknown and, importantly, our results and results obtained by others reveal that the knowledge and evidence acquired from transplantation models of other organs may not be universally applicable to the ocular niche.

Small animal models of corneal transplantation have a very prominent role in our attempts to understand the immune mechanisms (and not only those associated with transplantation) more comprehensively. Also, they help in assessing the efficacy of immunomodulatory drugs or approaches and in uncovering the possible mechanisms of their action. The thesis demonstrates a variety of possibilities these applications provide.

Appendix

AUTHOR´S PUBLICATIONS

Manuscripts

Sedláková K., Muckersie E., Robertson M., Filipec M., Forrester JV.: FTY720 in corneal concordant xenotransplantation; *Transplantation* 2005; 79: 297–303.

Sedláková K., Robertson M., Muckersie E., Duncan L., Filipec M., Forrester JV.: FTY720 Affects MHC Class II Expression on Lymph Node B Cells (submitted)

Sedláková K., Filipec M., Holáň V.: The effect of the suturing technique on corneal xenograft survival (submitted)

Sedláková K., Filipec M.: Corneal immunology: a perspective on factors affecting alio- and xenografts (submitted)

Chapters in books

Sedláková K., Dubská Z.: Choroideremie (case report) – accepted for publication

Sedláková K.: Mřížková dystrofie rohovky (case report) – accepted for publication

Sedláková K., Kalvodová B.: Pars plana vitrektomie u pokročilého nálezu makulární epiretinální membrány a diabetického makulárního edému (case report) - accepted for publication

E- Learning presentation - 1st Medical Faculty of Charles University, Prague

Sedláková K.: Vyšetřovací postupy II - Přední segment

Fichtl M., Martincová R., **Sedláková K.**, Růžičková E.: Etiopatogeneze a diagnostika glaukomového onemocnění

Sedláková K., Růžičková E.: Etiopatogeneze glaukomu (rozšířená volitelná verze)

Fichtl M., Martincová R., **Sedláková K.**, Růžičková E.: Klasifikace glaukomu

Fichtl M., Martincová R., **Sedláková K.**, Růžičková E.: Terapie glaukomu

Fichtl M., Martincová R., **Sedláková K.**, Růžičková E.: Glaukomový záchvat

- *Note.: The authors contributed equally to presented work*