Ocular responses to foreign corneal and tumor issue

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Ocular responses to foreign corneal and tumor tissue

Theodorus Huibertus van Essen

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Ocular responses to foreign corneal and tumor tissue

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‘The soul becomes dyed with the color of its thoughts’

Marcus Aurelius

Meditations, Book V, 171-175

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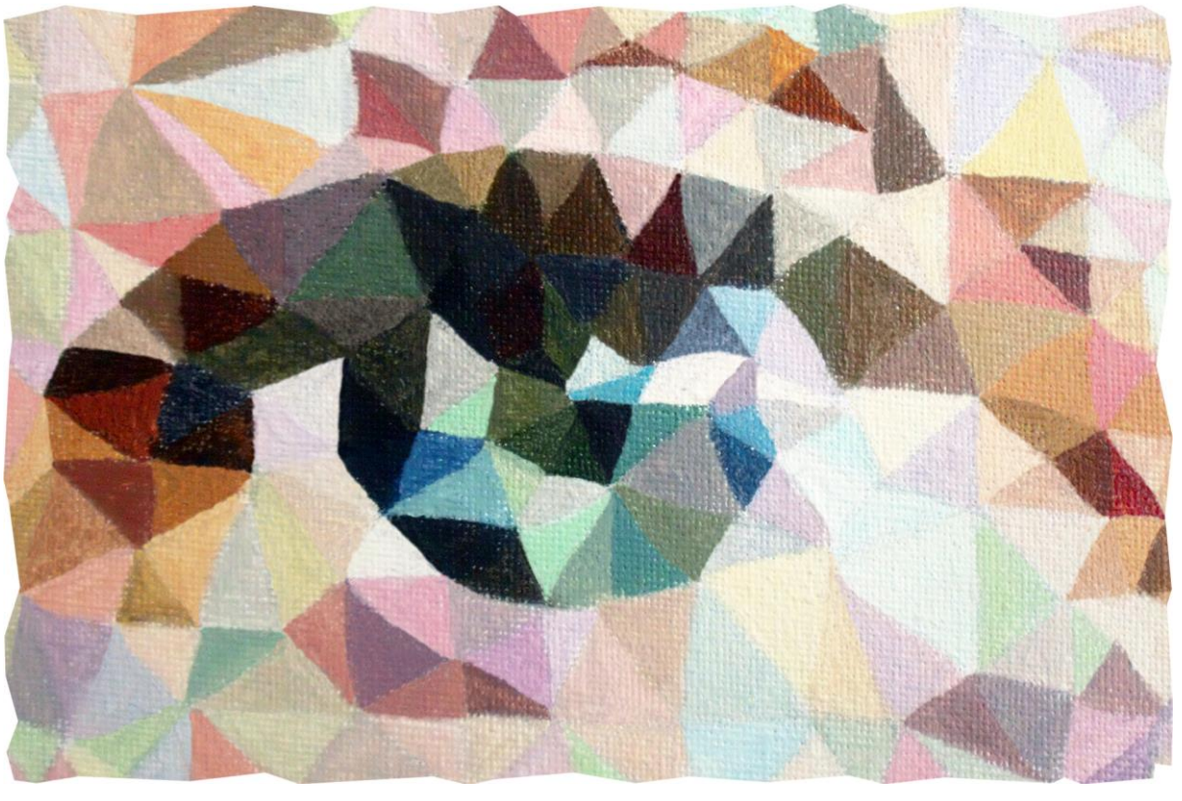
CHAPTER 1

General introduction



Introduction

Eyes are immunologically privileged, which means that the immune system behaves in a different way than elsewhere in the body: the ocular environment tends to suppress immune reactions. This immune privilege is the main reason behind the success of allogeneic eg. corneal transplantations, as it leads to inhibition of the rejection of transplants.

With regard to intraocular tumors, the ocular immune system places us for a paradigm: uveal melanoma (UM) is a tumor that develops inside the eye, but while the environment limits inflammation, tumors may contain a lot of infiltrating cells. However, in contrast with what one would expect in cancer, increased levels of infiltrating immune cells are associated with shorter instead of longer survival rates. In this case, a local immune response does not lead to tumor destruction, but local inflammation contributes to angiogenesis and is associated with the formation of metastases.

Several different factors determine the effectiveness of immune responses. Human leukocyte antigens (HLAs) for instance play a central role in most immune responses by presenting antigens, which may be derived from a transplant or from tumor cells, to the immune system. It may well be that the HLA levels are differentially regulated in different tissues and that this will provide us with an answer how the environment influences the behaviour of the immune system. In this thesis, I will discuss relevant immunological, environmental and other factors, first by summarizing the aspects that are critical for biocompatibility and acceptance of corneal implants, and secondly by discussing the way the immune system and the environment are involved in UM.

1. Immunology

1.1 Immune responses

The immune system protects the body against diseases by killing pathogens and tumor cells.

The innate immune system is the first active line of defense once our body's mechanical barriers have been breached. It reacts quickly, is non specific, and recruits immune cells to the site of danger using cytokines. The innate immune system subsequently activates the adaptive immune system by presenting antigens in the lymph nodes. The subsequent adaptive immune response is antigen-specific and has immunological memory, specific for each pathogen: when a pathogen enters the body a second time, specific immune cells will eliminate it faster than the first time. This adaptive immune system is the second active line of defense and consists of two groups of immune cells: B-cells and T-cells, both of which carry receptor molecules that recognize specific targets. The B-cell receptor is an antibody molecule that can recognize the pathogen in its free and generic form, while the T-cell receptor only recognizes processed pathogens presented as peptides by the HLA molecules of other cells.

1.2 Human Leukocyte Antigens (HLA)

HLA antigens are necessary for the induction of immune responses, as well as for the immunological effector phase, as they present target peptides to helper as well as effector T cells. HLA antigens are divided into two classes: HLA Class I and Class II. HLA Class I antigens are derived from the three classical loci HLA-A, -B and -C and the non-classical loci HLA-E, -F, -G, -H, -I and -J. HLA molecules are expressed on platelets and almost all nucleated cells, except most cells of the central nervous system. The HLA Class I molecules present intracellular peptides to cytotoxic CD8-positive T cells during the effector phase. HLA Class II consists of three main genetic loci: HLA-DR, -DQ and -DP. eClass II molecules are expressed on some immune cells such as B cells and activated T cells, and especially on antigen-presenting cells such as the DCs, macrophages, and monocytes, as well on endothelial cells and thymic epithelial cells. The Class II molecules present peptides from exogenous antigens to the CD4+ helper T cells in order to start the induction of an immune response. Cytokines can modify the level of expression of Class I and II molecules. The HLA genes are the most polymorphic genes in the human genome, providing a great diversity of HLA alleles, with each specific allele having the ability to present certain antigens better or worse than other alleles.

1.3 Immune privilege and corneal immune response

The immune privilege of the eye is caused by an immunological threshold, which is due to a variety of immuno-evasive and immuno-suppressive mechanisms. Because many tissues in the eye are amitotic and therefore incapable of regeneration, the eye is very sensitive to injury, including damage caused by inflammation. The eye deploys several tactics to reduce damage, affecting the innate and adaptive immune system.[1-6] Many blood vessels within the eye are non-fenestrated and contain tight junctions, restricting passage of inflammatory cells and macromolecules into the eye.[7] Membrane-bound molecules on the cells lining the interior of the eye induce apoptosis of invading activated T-cells.[8, 9]

In addition to these passive defenses against damage, an active system has been identified, known as Anterior Chamber-Associated Immune Deviation (ACAID): the aqueous humor of the eye contains soluble immunosuppressive and anti-inflammatory factors, such as TGF-β2, α-melanocyte-stimulating hormone (α-MSH) and vasoactive intestinal peptide (VIP). ([1, 10, 11] Exposure of antigen-carrying macrophages to these factors and their subsequent migration to the spleen leads to the induction of regulator T cells (Tregs) that suppress both the afferent and efferent arms of the immune response.[12] α-MSH inhibits delayed-type hypersensitivity, macrophages, and neutrophils and induces Tregs. VIP inhibits T-cell activation and proliferation, and inhibits the delayed-type hypersensitivity as well.

The human cornea consists of five layers: from the front to the back they are the epithelium, Bowman's membrane, stroma (containing the keratocytes), Descemet's membrane, and the endothelium. The cornea serves as the window of our eye. The function of the epithelium is to protect the cornea from the outside world, while the stroma provides the cornea with strength and

elasticity to withstand pressure and trauma. The endothelium serves to pump fluid out of the cornea; failure to do so results in corneal swelling and haze and severely-reduced vision. This makes the endothelium a very sensitive part of the cornea, especially because endothelial cells hardly regenerate.

The cornea exhibits some specific features which strenghten its immune-privileged status:

1) Absence of corneal lymph and blood vessels and blockade of lymph vessel formation, together with a relative lack of lymphatic drainage from the eye, ensures that antigens can only leave the eye via the aqueous drainage system into the bloodstream.[13] The aqueous outflow will carry antigens to the spleen, rather than the draining lymph nodes, which will then act as the primary lymphoid tissue.[14]
2) The compact architecture of the corneal stroma is believed to inhibit the infiltration of immune cells, while the blood-aqueous barrier prevents immunologically-active cells and factors from entering the ocular tissue.[14] Although functional antigen-presenting cells (APCs) are present in the peripheral and paracentral cornea, they are scarce and mostly immature in the healthy central cornea, resulting in weak local antigen presentation.[15]
3) Corneal tissue by itself is able to produce cytokines that inhibit T cell responses.[16]
4) Angiogenesis can occur, but several factors inhibit this. Endostatin is present and reduces VEGF receptor signaling. Intact epithelium expresses VEGF-receptor-3, which inhibits angiogenesis, possibly by catching the free VEGF molecules. Soluble VEGF receptor-1 in the extracellular matrix performs in the same way by binding VEGF-A before it can induse angiogenesis.[17]
5) The epithelium and endothelium express Fas-ligand, which can induce apoptosis of T-cells and neutrophils,[18] and Programmed Death-Ligand 1 (PD-L1) which inhibits T-cell proliferation, interferon-gamma (IFNG) secretion and induses apoptosis.[19]

In the clinical setting, a local immune response elicited for example by surgery, is further tempered by topical corticosteroids applied to the cornea post-operatively or by oral immunosuppressives.[20]

1.4 Human cornea allograft transplantation

Due to its immune privilege, cornea allograft transplantation is one of the most successful types of solid organ transplantation, with one-year graft survival rates above 90%.[21, 22] However, the 15-year graft acceptance rate is around 55%, which equals survival rates of other forms of solid organ transplantation.[21] Graft failure is defined as the irreversible loss of a graft's clarity. This can be due to immunological rejection, endothelial dysfunction, surgical trauma, infection or secondary glaucoma.[23] The most common cause of transplant failure is rejection, accounting for 30% of the failures.[21, 22] Rejection occurs because the host's immune system attacks and gradually destroys the transplant. Several approaches can be exploited to reduce the rate of immune rejection, which are described in Chapter 1.

1.5 Alternatives for human cornea transplantation

There is a worldwide shortage of human donor corneas, especially in developing countries.[24, 25] Furthermore, human donor corneas are expensive (around 3000 American dollars [26]) and not suited for a hostile host environment, such as inflamed or severely neovascularized corneas. Therefore, research has taken place for over 100 years to discover alternatives for human donor corneas,[27] and several alternatives exist. I will discuss the most well-known of these alternatives, which are either currently in use, or under investigation.

1.6 Boston Keratoprosthesis

The Boston Keratoprosthesis (BKpro), FDA approved since 1992, is a corneal implant, consisting of a front plate containing an optical cylinder and a plastic or titanium back plate that locks the optical cylinder in place. Between the front and back plate, a human donor cornea is inserted.[28] Indications for use of the BKpro are cases of corneal blindness with failed grafts, significant corneal neovascularization, limbal stem cell deficiency, autoimmune disease or chemical injury.[28] These patients should however have a healthy ocular surface in order to retain a bandage lens postoperatively.[29] The BKpro still needs a human donor cornea, with its risk of rejection, and is rather expensive, although the developers of the BKpro are working towards cost reduction.[30]

1.7 Osteo-Odonto-Keratoprosthesis

The Osteo-Odonto-Keratoprosthesis (OOKP), described first in 1963,[31] uses a tooth root and alveolar bone to support a centrally-implanted optical cylinder. For this, a labor-intensive, two-stage surgery is performed.[32] First, the ocular surface (epithelium of the cornea and conjunctiva) is removed and replaced with buccal mucosa. During the same surgical procedure, the tooth is harvested and the optical cylinder is placed in its center, after which the tooth-optic is placed subcutaneously or submuscularly in the orbito-zygomatic area on the contralateral side to attain a fibrovascular coating. Secondly, the implant is explanted 2-4 months later, and sutured in place over the cornea, with the posterior optic protruding into the corneal opening, and the anterior optical cylinder protruding through the buccal mucosa covering the bulbus.

Indications are bilateral corneal blindness due to several ocular and systemic pathologies such as end stage Stevens-Johnson Syndrome. The surgery however is performed usually only in one eye, keeping the other eye as a spare.[32]

Although being the most successful and well-retained keratoprosthesis,[33] the complexity of the surgery, its high cost, and it being indicated only for high risk patients, make it no real alternative to human donor cornea transplantation.

1.8 Collagen-based cornea

Collagen-based artificial corneas form another alternative for human corneal transplantation. They are largely three-dimensional scaffolds made from biomaterial, mimicking as well as possible the

human corneal stroma, which should upon grafting ultimately be repopulated by the host's corneal cells.[34-36] For anterior lamellar approaches, an acellular scaffold can be used, thereby reducing the chance of rejection. The most promising of these is the synthesized recombinant human collagen type III scaffold of Fagerholm, et al.[35] A drawback of this synthesized scaffold is the difficult fabrication process;[37] another one is the need for better tensile strength in order to be able to perform continuous suturing instead of overlaying sutures.

Other fabricated constructs for regenerating the human cornea have not yet reached the clinical stage. Collagen-based alternatives for posterior lamellar or full-thickness approaches need viable endothelial cells on the posterior side in order to prevent stromal swelling,[38, 39] or other means to prevent this swelling. The alternative posterior approaches will not be discussed here.

1.9 Decullarized cornea

Decellularized human corneas are basically also collagen-based scaffolds but, as one still needs a human donor cornea, their use does not decrease the lack of need for human tissues. A decellularized porcine corneal stroma is another option that is being explored [40] , but porcine tissue confers a risk of transmitting animal diseases.[41] Screening may reduce that risk, but would come at a price.[41]

1.10 Stem cell-based approaches

Stem cell-based approaches are being explored to regenerate part of or even the whole cornea. The biggest challenge is to have the human cornea stromal cells, the keratocytes, secrete specific types of collagen as the orthogonally-arranged multilayered lamellae are needed for the corneal transparency and strength. For this, human corneal stromal stem cells (hCSSCs) are being used, which under specific culture conditions can differentiate into cells similar to keratocytes, which secrete an extracellular matrix that mimics the corneal stroma.[42, 43] These hCSSCs may also have potency to restore the disrupted collagen fiber organization of scarred corneal stroma,[44] or can induce stromal healing without scar formation when implanted directly after damage.[45]

1.11 Other scaffold materials

Asides from the keratoprosthesis, collagen scaffolds and stem cell approach, other materials are also being explored as scaffolds for corneal cells in order to regenerate the human cornea. Among these materials is the electrospun gelatin nanofiber scaffold of Tonsomboon et al.,[46] a two-component scaffold with a central core of poly(ethylene glycol)/poly(acrylic acid) (PEG/PAA) covered with collagen type I and a microperforated poly(hydroxyethyl acrylate) (PHEA) hydrogel skirt with the surface covered with collagen type I.[47] Also, scaffolds made from silk are being developed.[48]

1.12 Requirements of an artificial cornea

As described above, the cornea consists of five main layers. The layer that is in contact with air is the epithelium, which is gradually replaced every 7-14 days by proliferation and subsequent differentiation of the basal cells. The basal cells themselves are also constantly replaced by cells origination from the limbal stem cell niche, and migrate from the peripheral cornea towards the center.[49-51] The stroma makes up 90% of the total corneal thickness, which measures around 520-540 um at the center,[52, 53] and up to around 610 um at the periphery.[53, 54] It is a mainly acellular layer with only 3-10% of its volume consisting of quiescent keratocytes.[55] The cornea's transparency and its strength are due to the specific arrangement and uniformity of its collagen fibers and the dehydration state.[56-59] The collagen fibers are mostly type I, but type V, VI and XII are also present.[60] The diameter of these fibers is a uniform 31-34 nm,[55, 61, 62] and their diameter is around 22.7 nm ± 1.8 nm in a study of corneas immediately fixid after surgery,[59] with an interfibril spacing of around 20 nm, maintained by collagens and proteoglycans.[59] This, together with the cornea being the window of the eye, brings us to the requirements of an artificial cornea based on a collagen scaffold, as shown in Table 1.

We decided to focus on an anterior approach, leaving the endothelium intact. In Chapter 2, we focus on the first results of a fish-scale derived collagen matrix (FSCM), a scaffold with high water content and a good oxygen permeability as the basis for corneal regeneration.[63]

We describe the results of light scatter and transmission, and the first short-term in vivo experiments in rats.

Table 1 Requirements of an artificial cornea

Light transmission	± 91% [64]
Forward light scatter	range 0.9 (healthy young) - 1.5 (old) log(s)[65]
Water content	± 78% [66]
Oxygen permeability	± 29 x 10^{-11} (cm^2 x ml O_2)/(sec x ml x mmHg)[67]
Glucose permeability	± 2.5 - 3.0 x10^{-6} [68]
Albumin permeability	albumin permeability ± 2.1 - 4.1 x10^{-8} [69]
Young's Modulus (elasticity)	± 3-13 MPa [70]
Tensile strength	± 3.8 MPa [71]
Suturable	
Not immunogenic	
No immune sensitization	
Facilitates reepithelialization on anterior side	
Allows tissue incorporation or tissue attachment	
Facilitates endothelium attachment on posterior side	**In case of penetrating keratoplasty*

In this paper, we put emphasis on the implantation technique and immune responses. In Chapter 3, we describe the in vitro results of co-cultures between cornea cells and the FSCM, and additional in vivo experiments with longer follow up. Using in vivo experiments, we analyze the behavior, immune response and possibility of sensitization against the FSCM, and compare the results to another matrix already used in ocular surgery, and to sham surgery. Additionally, we determine the tensile strength and glucose permeability of the fish scale collagen matrix.
A review of the role of the immune system and matching for the major histocompatibility antigens in corneal transplantation follows in Chapter 4.

2. Uveal melanoma

While local immune privilege allows acceptance of corneal transplants, lack of an effective immune response against tumor cells may play a role in the outgrowth of malignant melanoma cells inside the eye. UM is the most common intraocular tumor in adults with an incidence that ranges from 4.3 to 10.9 per million,[72-75] with the higher incidence being in areas populated by whites.[76] Up to 50% of the patients with large tumors that need enucleation may develop metastases which are almost always fatal.[77] The 5-year survival of all cases remains around 69-78%, death is usually due to metastasis.[72, 78] Over the last decades, survival has not improved.[73]
UM has its origin in the melanocytes of the uveal tract, which consists of the choroid, ciliary body and the iris. Most tumors are located in the choroid (86%).[76]
Risk factors for developing UM are congenital ocular and oculodermal melanocytosis (nevus of Ota) with a lifetime risk of developing UM of 1 in 400.[79] Other risk factors for the development of UM are light eye color, fair skin, and inability to tan, [80] , and the presence of a veal nevus. Uveal nevi occur in 5-8% of whites, but only 1 in 8845 nevi transform into UM.[79] However, 18% of extraordinarily large nevi (≥10mm in diameter) progresses into melanoma over 10 years.[81]
Once a patient has been diagnosed with primary UM, the chance to develop metastasis can be calculated using several parameters. UM metastasises haematogenously unless it invades the conjunctiva, in which case it can spread to regional lymph nodes; this is extremely rare.[82]
Prognostic factors include largest basal diameter, thickness of the tumor, ciliary body involvement and extrascleral extension.[82] Other prognostic factors are cell type (epithelioid or spindle), non-random chromosomal aberrations or the gene-expression profile of the tumor. A shorter survival is seen in patients with epithelioid cell type, loss of the whole chromosome 3, gain of the long arm of chromosome 8, and the gene-expression profile class 2 (as based on 15 genes).[83-85] Several factors are combined into the TNM-classification which is based on the size of the primary tumor, involvement of the ciliary body, extraocular extension, and the presence of metastases.[86] Adding chromosome status to the TNM class adds precision.[87]
Local treatment of the primary tumor, in case of absent metastasis, has good results, but it seems to have no impact on the metastasis rate. Possible treatments of the primary tumor include brachytherapy (for tumors ≤ 10 mm thickness) and proton-beam radiation, which carry equal

outcomes with enucleation.[88-90] When the tumor is too large for radiation, enucleation remains the treatment of choice.[90]

There is no effective treatment of metastasized disease, although several modalities have been tried and are still under investigation.

2.1 Link between HLA, inflammation and UM

It is an intriguing finding that in UM, the presence of an increased level of infiltrating immune cells does not prevent, but seems to stimulate tumor progression. If we solve the mechanism behind this phenomenon, we may find therapies to attack and destroy the metastasis and be able to prevent progression and subsequently, the patients' death.

It is known that cancer cells can use immune evasion in order to survive.[91] It has been assumed that the most common method a tumor deploys to escape from T-cells is reducing its antigen expression by down-regulating the expression of its HLA molecules.[91, 92] This is certainly not the case in UM, as a higher death rate is associated with an increased HLA expression.[93-95]

However, other mechanisms exist, such as alterations in the expressed subtypes of HLA molecules, or, for example, expression of non-classical instead of classical HLA molecules. Natural Killer (NK) cells specifically attack cells without HLA Class I, and tumor cells with a high HLA Class I can evade NK-cell-mediated killing while coursing through the blood. Other escape mechanisms are immune evasion through defect death receptor signaling, lack of co-stimulation or the secretion of immunosuppressive cytokines and attraction of immunosuppressive T-cells.[91] An example of creating a favorable immune microenvironment for tumor growth is the attraction of tumor-associated macrophages (TAMs), of which the M2 type is known to play a role in promoting angiogenesis and inhibiting immune responses.[96]

In UM, important immunological parameters associated with prognosis are lymphocyte ([97] and macrophage infiltration.[98] High numbers of tumor-infiltrating $CD68^+$ and $CD163^+$ (M2) macrophages are associated with an unfavorable prognosis,[96, 98, 99] and $CD68^+$ (M1) macrophages have been associated with increased HLA Class I and II expression.[100]

2.2 HLA genotype and UM

We have focused mainly on the role and function of HLA in UM. We already know that in UM, tumors which metastasize have an increased expression of HLA molecules compared to those which do not.[100] Other studies have shown that HLA polymorphisms may mediate susceptibility to certain cancers,[101, 102] and a possible connection between HLA-B40 or B44 with metastasis in UM has been suggested.[103, 104] There are also several specific associations between HLA antigens and ocular diseases, of which especially those in which pigment is somehow involved are of interest. Birdshot Chorioretinopathy (BCR), which is characterized by multiple hypopigmented chorioretinal lesions, is associated with HLA-A29.[105] Vogt-Koyanagi-Harada syndrome (VKH), a bilateral, chronic, diffuse panuveitis in which late stage depigmentation of the fundus occurs, has a genetic association with

HLA-DR4.[106] Using this as a starting point, we set out to investigate, whether a person's specific HLA genotype may predict the amount of HLA expression in UM or may be indicative for the level of inflammation in this malignancy.

2.3 HLA regulation, prognostic factors and UM

The genes encoding the HLA Class I and Class II antigens are located on chromosome 6p. Generally, chromosomal gain leads to an increased expression of the genes on that chromosome in tumors.[107, 108] This places us for an intriguing paradox in UM, as gain of chromosome 6p in tumor cells is associated with a good prognosis, while an increase in HLA Class I and II expression is associated with a poor prognosis.

Several other factors beyond gene dosage influence the level of HLA expression. First of all, to be functional, the HLA molecules should reach the cell surface. This requires a properly-functioning peptide-loading system.[109] Second, transcription of the HLA genes is regulated by several genes, such as *NLRC5* and *CIITA*. *NLRC5* plays a crucial role in the transcriptional regulation of HLA Class I genes,[110] and *CIITA* in the transcriptional regulation of the *HLA Class II genes,*[111] while it is also involved in *HLA Class I* transcriptional activation.[112] The promoters *NLRC5* and *CIITA* are in turn influenced by, amongst others, the interferon-regulatory factor 1 (IRF1).[113] *CIITA* is silenced by EZH2 (Enhancer of Zeste Homologue 2, a Polycomb Repressive Complex 2 subunit; chr7q).[114]

Not only these transcriptional regulators influence HLA Class I and Class II expression, but also external influences. Interferon-gamma (IFNG) stimulation is known to increase the level of HLA Class I and Class II in UM cell lines.[114, 115] Down-regulation of HLA expression on cell lines may be induced by tumor growth factor beta (TGFB).[115, 116]

Without HLA molecules, T-cells cannot react to and subsequently destroy their target cells.[117] This underlines the importance of determining whether HLA expression in UM cells functions properly, and how it is regulated. We therefore investigated in primary enucleated tumors, instead of cell lines, whether chromosomal dose effects or specific known regulators influence HLA gene or protein expression in UM. The outcome is described in Chapter 6. We also analyzed the influence of the genes encoding for the peptide-loading system molecules. Lastly, we assessed the possible influence of the microenvironment on HLA gene expression by comparing expression levels in human primary or metastatic UM with their corresponding xenografts placed in mice, which lack tumor-infiltrating leukocytes.

2.4 M1 and M2 macrophages

As said earlier, UM creates a tumor promoting microenvironment, in which M2 macrophages play a important role. TAM promote tumor growth, angiogenesis, metastasis and induce immunosuppression. These functions contrast with the M1 macrophages which are known to have anti-tumoral activity.

The microenvironment in which the macrophages reside induces the polarization towards either an M1 or M2 type. The M2 macrophages are induced by interleukin-4 (IL-4), IL-10 and IL-13,[118] (as well as macrophage colony-stimulating factor (M-CSF) and CC chemokine ligand-2 (CCL2).[119, 120] M2 macrophages express HLA Class II at a lower level than M1 macrophages, [121] and are insufficient for antigen presentation.[122] . They produce also IL-10 themselves as wel as TGF-β, and help to maintain the tumor promoting environment.

The tumor promoting environment is a result of the complex interplay between tumor cells, regulatory T-cells and macrophages, which possibly can be influenced for example by immunotherapy. This may skew the polarization towards the proinflammatory M1 type.[123]

2.5 BAP1 and UM

To broaden our scope, we looked at new prognostic factors for UM. This could provide us with new clues of how UM evade destruction by the immune cells. Harbour et al. demonstrated that loss of one copy of chromosome 3 in combination with inactivating mutations in the gene encoding BAP1 (BRCA1-associated protein 1) on the remaining copy of chromosome 3, is associated with metastasis.[124] BAP1 exerts a tumor suppression function, and is a deubiquitinating enzyme of the polycomp-group proteins of transcriptional repressors.[125-127] We determined how expression of BAP1 at the mRNA or protein level was related to prognosis (chapter 7). A subsequent study has shown that loss of BAP1 is strongly associated with tumor infiltration with lymphocytes, suggesting that BAP1 is an immune response regulator.[128]

3. This thesis

This thesis looks at the effect of the ocular environment on two important areas: can we develop a new biocornea that may be used to replace a damaged human cornea, and can we find out how to modulate the cells of UM to find ways to prevent or treat metastases? The link between the two is the role of HLA antigens in rejection and inflammation, as these are essential for inducing an immune response as well as for the effector phase.

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CHAPTER 2

A Fish Scale–Derived Collagen Matrix as Artiftcial Cornea in Rats: Properties and Potential

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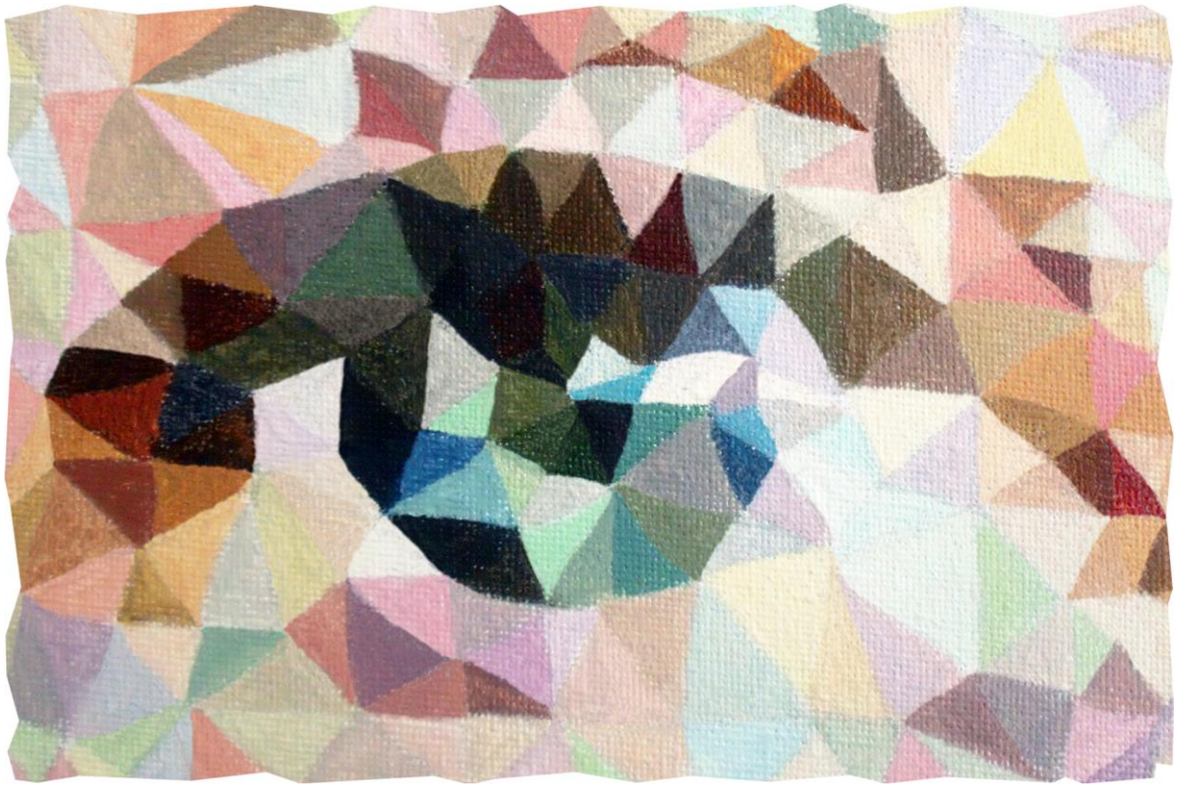

Abstract

Purpose: A fish scale–derived collagen matrix (FSCM) is proposed as an alternative for human donor corneal tissue. Light scatter and light transmission of the FSCM were measured and compared with human cornea, and its short-term biocompatibility was tested in a rat model.

Methods: Light scatter was determined with a straylight measuring device, whereas light transmission was measured using a broadband absorption spectrometer. For evaluation of the biocompatibiliy, three approaches were used: the FSCM was implanted as an anterior lamellar keratoplasty (ALK), placed in an interlamellar corneal pocket (IL), and placed subconjunctivally (SC). Transparency, neovascularization, and epithelial damage were followed for 21 days. Morphology and cellular infiltration were assessed histologically.

Results: The amount of scattered light was comparable to that seen in early cataract and the percentage of light transmission was similar to the transmission through the human cornea. Implantation of the FSCM as an ALK led to mild haziness only, not obscuring the pupil, despite the development of neovascularization around the sutures; IL placement led to a moderate haze, partly obscuring the pupil, and to (partial) melting of the anterior corneal lamella. The SC group exhibited local swelling and induration, which decreased over time. Histology showed a chronic inflammation varying from mild and moderate in the ALK and IL group, to severe in the SC group.

Conclusions: In spite of technical difficulties, it was feasible to use the FSCM for ALK, whereas IL placement led to melting of the anterior lamella. Further studies are necessary for better understanding of its immunogenicity. The light scatter and transmission data show that the first version of this FSCM is comparable to human cornea tissue in this respect.

Introduction

Corneal disease is a major cause of blindness worldwide, second only to cataract. Globally, more than 10 million individuals are bilaterally blind due to corneal pathology and even more unilaterally.[1] Corneal transplantation is presently the only option to restore vision in these patients. Full-thickness corneal transplantation is one of the most successful forms of tissue transplantation and has been performed since 1905. One year after transplantation, the success rate is excellent in low-risk cases (avascular corneas). However, at longer follow-up, the overall acceptance of the grafts declines, particularly in high-risk cases.[2–5] The worldwide demand for human donor corneas, however, exceeds the world's supply, especially in developing countries.[6] Artificial corneal substitutes have emerged to counter this shortage and overcome the disadvantages of human donor corneas, including immune rejection. These corneal substitutes range from completely synthetic prostheses, which primarily aim to restore the cornea's refractive function,[7,8] and tissue-engineered cell-based constructs,[9] to hydrogels and scaffolds that facilitate the regeneration of the host tissue.[10–12]

Although much progress has been realized, keratoprostheses have not reached widespread use.[8] At the moment, they are too expensive and complex for routine use in developing countries, where the need for implants is highest. Alternatively, biological explants are being developed and tissue-engineered corneal epithelial cell sheets have been successfully transplanted in patients.[13–15] As corneal pathology often extends beyond the epithelium and affects the corneal stroma as well, replacement of the corneal stroma is usually necessary. To replace the corneal stroma, a cell-based construct or polymer scaffold of sufficient thickness is needed.

Ideally, such a scaffold for corneal regeneration allows reepithelialization, endothelialization, and repopulation with interstitial cells and nerves, preferably all of the patient's own origin. However, human corneal endothelium hardly regenerates. For many corneal opacities without endothelial involvement, procedures that preserve the recipient's endothelium are used, such as anterior lamellar keratoplasty (ALK) and deep ALK (DALK). These anterior keratoplasties result in much better graft survival than the full-thickness grafts in penetrating keratoplasty (PK).[16–18] The anterior lamellar approach is therefore a logical starting point for scaffold-based corneal regeneration. In 2010, this approach was studied with a biosynthetic implant in a phase I clinical trial with 10 patients. Corneal regeneration with restored vision and sensitivity was found after 24 months of follow-up,[19] proving potential of this concept. The biosynthetic implant was synthesized from human recombined collagen type I, which is an elaborate and rather expensive procedure, and despite the claimed success in this study, no new trials have started so far. Collagen scaffolds that already exist in nature may reduce the cost of fabrication and promise a sufficient resource for clinical transplantation even in developing countries. A decellularized porcine corneal matrix as a xenographic scaffold for corneal regeneration has been studied for several years by several research groups, as it closely resembles the human corneal stromal organization.[12,20,21] Indeed, such porcine matrices may offer a relative inexpensive and widely available alternative to human donor corneas. Results from clinical trials are

not yet available, but a first phase I clinical trial is currently running.[22] Collagen matrices that are even more widely available, easier to harvest, and at lower expense, therefore definitely offer an interesting alternative, and this is the topic of our study.

Here we present the results of a matrix made from naturally occurring collagen type I and obtained from scales of the tilapia fish. Tilapia (Oreochromis mossambicus) are farmed for consumption under controlled circumstances[23] and the specific size of the fish can be selected on harvesting. Their scales consist of parallel-arranged collagen fibers packed in layers oriented approximately 90 degrees,[24] mimicking the human corneal stroma. This scaffold supports the growth of corneal cells and is highly permeable for oxygen.[25] Moreover, collagen sponges fabricated from reconstituted collagen fibers from tilapia scales produce only rare inflammatory responses in vivo.[26]

To investigate the suitability of this fish scale–derived collagen matrix (FSCM) for use as an artificial cornea, the light-scatter and light-transmission properties were examined. Also, in vivo studies were performed, using the rat keratoplasty model, widely employed for corneal transplantation studies.[27] We investigated the short-term biocompatibility and handling of the FSCM in three different surgical rat transplantation models.

Methods

Fish Scale-Derived Artificial Cornea

A ±250 µm-thick decellularized and decalcified fish scale–derived extracellular matrix (ECM), consisting of collagen type I, was used for implantation (7-mm diameter, 0.2–0.3 mm thickness, P09011001; Aeon Astron Europe, Leiden, The Netherlands) (Fig. 1).[25] In short, fresh tilapia scales were cleaned in distilled water and cellular components were removed using a four-step detergent and enzymatic extraction process as developed by Courtman et al.[28] Acetic acid was used to increase pore size and porosity and nitric acid to decalcify the fish scales. The decellularized and decalcified scales were rinsed with 70% ethanol and stored until use in sterilized PBS at 48C.

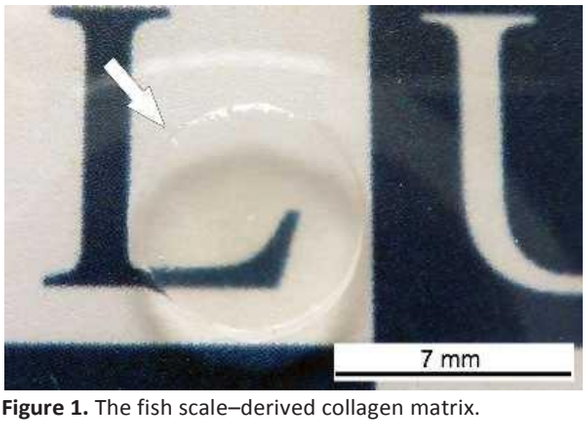

Figure 1. The fish scale–derived collagen matrix.

Top Pattern, Light Scatter, and Transmission

Phase-contrast images (Axio Observer.A1; Carl Zeiss AG, Jena, Germany) were taken of the prepared fish scale–derived collagen scaffold and used to create a composition photograph in Adobe Photoshop (CS3 Extended, version 10.0; Adobe Systems Incorporated, San Jose, CA) to visualize the whole top surface. Scanning electron microscope images were taken of the cutting edge of the prepared FSCM and of the micro pattern on the top surface.

To measure forward light scatter, we chose a similar approach as is used clinically to assess the functional effect of forward light scatter in patients.[29] This way, the ex vivo results can directly be compared with clinical in vivo results. We applied a psychophysical technique known as ''compensation comparison'' and the outcome value is the straylight parameter's.[30] This technique is implemented in a commercial instrument (C-Quant; Oculus GmbH, Wetzlar, Germany) for clinical use, measuring the light scatter between 5 and 10 degrees, which has proved representative for the total amount of straylight (light scatter ≥1). We used this instrument, with a slight adaption, to assess forward light scatter from physical samples.[31] In short, two stimuli of the compensation comparison method, straylight flicker and comparison flicker, are presented to and compared by the subject simultaneously. To exclude the influence of light scatter provoked by the observer's eye and to measure only the light scatter caused by the matrix, the straylight flicker source itself was shielded in such a way that it illuminated only the tested sample.[31] Three FSCMs (P11251101; 8.0 ± 0.8 mm [diameter], 0.25–0.35 mm [thickness]) were put on a test glass, one drop of PBS was used to prevent dehydration, and a cover glass was put on top. A black, opaque disk, with a central hole of 6.5-mm diameter, was mounted over the test glass, leaving only the FSCM visible. The test glass, with the FSCM and black opaque disk, was put in the ocular of the C-Quant device to measure the light scatter. Three measurements per FSCM were performed. A holder containing PBS without an FSCM acted as a control experiment.

To measure light transmission, three FSCMs (P11251101; 8.0 ± 0.8 mm [diameter], 0.25–0.35 mm [thickness]), placed in a sample holder and kept hydrated with PBS, were studied by a broadband absorption spectrometer, covering the visible and near infrared spectrum with a spectral resolution of 0.55 nm. Details are available from Bouwman et al.[32] The setup comprised a light source (LOT Oriel Xe-arc; 300 µm filament; LOT-QuantumDesign GmbH, Darmstadt, Germany) and spectrometer (Andor Shamrock SR-303i; Andor Technology PLC, Belfast, UK). The light source emitted white light with a homogeneous intensity pattern in the 200- to 760-nm region, fully covering the wavelength domain relevant to the human eye. This light was guided via a diaphragm through the center of the FSCM, which was placed 15 cm from the 10 µm wide inlet of the spectrometer. The sensor of the spectrometer measured light only in the horizontal plane. The maximum angle at which light could enter the spectrometer was 0.004 degrees. The spectrometer measured the direct light transmission, as straylight is defined as light being scattered at 1 or more degrees. All measurements were taken relative to atmospheric air and normalized for background light. Normalization and measurement of

transmission values for empty space were performed directly before each individual measurement. A holder containing only PBS was used to correct for light absorption or scatter caused by the holder itself. The light transmission of the visible spectrum was measured in steps of 0.56-nm wavelength and compared with the total light transmission of the human cornea, using the formula of van den Berg and Tan.[33]

Suturing

Two human donor eyes, obtained from the Euro Cornea Bank (Beverwijk, The Netherlands), were used to test the suturability of the scaffolds with nylon 10/0 sutures (nr. 8065 198001; Alcon B.V., Gorinchem, The Netherlands). In short, two FSCMs of 6-mm diameter were sutured into human corneas, 1 day postmortem, by placing 12 interrupted sutures in one case and continuous suture in the other, including knotting and burial of the knots. Severity of tearing due to the suturing was observed.

Animals

Eighteen male Fischer 344/DuCrl albino rats (Charles River Laboratory, L'Arbresle Cedex, France), each weighing between 280 and 336 g, were used for ocular implantation with permission of the Animal Ethics Committee of the Leiden University Medical Center. All animals were treated in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. They were given 1 week to acclimatize.

Implantation

The rats were divided into three groups of six animals, and in order to determine biocompatibility, scaffolds were placed in the anterior eye. In the first group, the FSCM, with the pattern on top, was placed as an ALK. In the second group, the FSCM was placed in an intralamellar pocket (IL), and in the last group it was inserted subconjunctivally (SC). The rats were anesthetized with isoflurane, with the addition of a topical drop of oxybuprocain and a subconjunctival injection of 20 µL bupivacain. Prior to implantation, the FSCM was soaked for 5 minutes in antibiotics (polyspectran containing gramicidine, neomycine, and polymyxine B).

ALK was performed by trephination of the right eye using a 3-mm trephine. The anterior tissue was removed using an air bubble, lamellar dissector, and scissors. The FSCM was cut to a diameter of 3.2 mm, inserted, and attached with eight nylon 10/0 sutures (nr. 8065 198001; Alcon B.V.). The surface of the FSCM was carefully leveled to line up with the native epithelium.

For the IL group, a nonpenetrating incision was made with a 158 stab knife and a pocket was created using an air bubble and a lamellar dissector. An FSCM with a diameter of 2.7 mm was inserted and the pocket was closed with two 10/0 nylon sutures.

SC implantation was performed by making an incision with a 158 stab knife. A blunt spatula was used to create a subconjunctival pocket, into which an FSCM with a diameter of 2.7 mm was inserted; the pocket was closed with two 10/0 nylon sutures.

Dehydration of the eye during surgery was prevented by regular wetting of the eye with special eye washings (balanced salt solution, 907553; Pharmacy LUMC, Leiden, The Netherlands). Directly after surgery, 1% chloramphenicol ointment (Chloramphenicol-POS 1%, 10 mg/g; Ursapharm Benelux B.V., Helmond, The Netherlands) was applied. In all cases, the unoperated left eye served as a control and was kept hydrated with gel (Vidisic Carbogel, carbomer 2 mg/g; Tramedico B.V., Weesp, The Netherlands) to prevent dryness during surgery.

In Vivo Observation

The animals were observed in the 3 weeks following implantation. Ocular drops with corticosteroids and antibiotics (Tobradex, containing dexamethasone and tobramycine; Alcon Cusi SA, Barcelona, Spain) were applied in the ALK and IL groups, once daily during the first week and every other day during the second week. All drops were stopped at the 2-week follow-up. The SC group did not receive any drops postoperatively.

At 2, 7, 13, and 21 days after implantation, all animals were examined with a microscope to judge neovascularization, transparency, and clinical signs of inflammation, such as conjunctival redness or purulent secretion. Corneal neovascularization was numerically scored from 0 to 5, with 0 no vessels, 1 growth of vessels at the limbus, 2 vessels reaching the sutures/FSCM, 3 vessels present underneath the FSCM, 4 vessels entering the FSCM, and 5 vessels present throughout the whole FSCM. Transparency of the cornea was assessed using a grading scale as previously used by Hackett et al.,[34] with 0 = transparent, 1 = a mild haze not obscuring the pupil, 2 = a moderate haze partially obscuring the pupil, and 3 = an opaque area totally obscuring the pupil.

Histopathological Evaluation

After euthanizing the 16 rats with carbon dioxide, the eyes were enucleated and fixated with Davidson solution (composed of glacial acetic acid, ethyl alcohol, and buffered formalin) and then dehydrated and embedded in paraffin. Sections were cut on a microtome (Leica RM2165; Leica Microsystems GmbH, Wetzlar, Germany) at 5 lm and stained with hematoxylin and eosin (HE) for histological examination. All eyes were analyzed for the organization of the epithelium, stroma, and endothelium, and scored semiquantitatively for infiltration of immune cells and ingrowth of corneal cells into the FSCM. The immune infiltrate was characterized based on morphology.

Statistical Analysis

All statistical analyses were performed using a statistical software program (SPSS for Microsoft Windows, version 17.0.2; IBM SPSS Statistics, IBM Corporation, Chicago, IL). The $\chi 2$ trend test was used for assessing differences between the groups regarding corneal neovascularization and

opacification, and leukocyte infiltration. Statistical significance was assumed for resulting P values less than 0.05.

Results

Top Pattern, Light Scatter, and Light Transmission

The whole top surface of the FSCM was visualized with a composed phase-contrast image. The micro pattern on top of the FSCM differed per area, with roughly one quarter having a spider-web appearance with micro ridges and channels, two quarters exhibiting circular running ridges and channels, but without intersecting lines, and the last quarter consisting of spikes (Fig. 2a). SEM images of the FSCM confirmed these findings (Figs. 2b–d). The micro ridges were wavelike in shape, ±5,7 µm high, and ±3.4 µm wide. The channels in between were indeed ±30 µm wide (Fig. 3).[25]

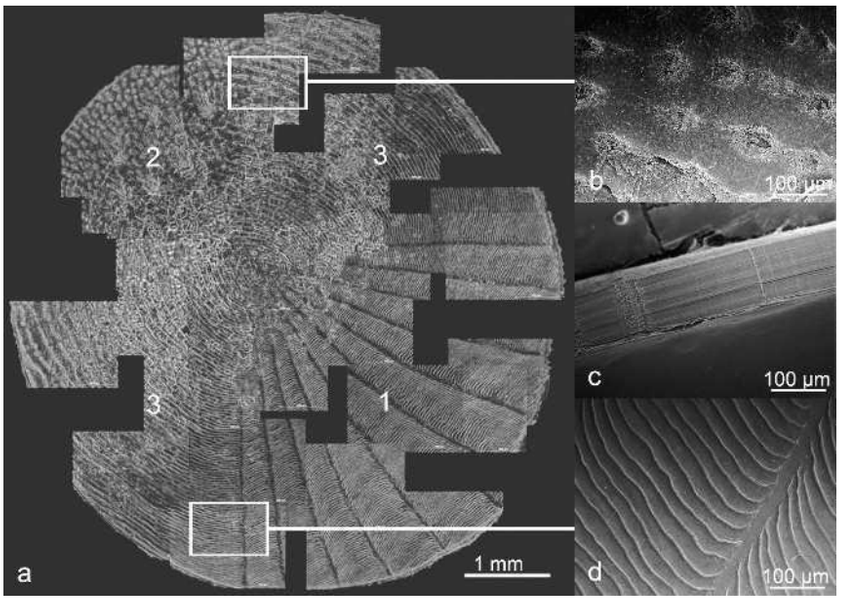

Figure 2. Composition of several phase-contrast images showing the distribution of the micro pattern with the spokes and interconnecting channels in one quadrant (a-1), the spikes in the opposite quarter (a-2), and the circular running lines in the area (a-3) between those two quadrants. Detailed SEM-images depict the transitional area between the circular lines and spikes (b), between the lines and spokes (d), and a cross section showing the multiple layers (c).

The amount of straylight was measured for three FSCMs using the compensation comparison method. Log values for forward light scatter caused by the holding device were beyond the lowest measurable value for the C-Quant device (log [s] < 0.40) and were considered to be negligible. The mean light scatter of the three FSCMs was log (s) = 1.62. All results are listed in Table 1.

Direct light transmission of the visible spectrum was measured on another three FSCMs. The mean direct light transmission of the three measured FSCMs, corrected for reduction of light transmission

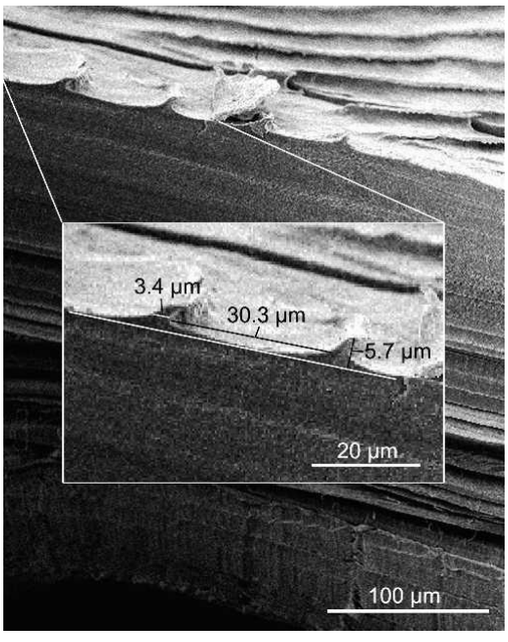

Figure 3. Cross section of the FSCM showing the dimensions of the circular running ridges on the surface.

caused by the PBS-filled holder, amounted to 89%, 97%, and 85% and showed corresponding curved graphs (Fig. 4a). The three measurements were grouped and compared with the total light-transmission curve of the human cornea (Fig. 4b). The human corneal light transmission values were within the SEM of the FSCM light-transmission values. The mean direct light transmission of the FSCM was 90%, whereas the total light transmission of the human cornea was 91%.[33]

Table 1. Forward Light Scatter Results in Log(s) With Oculus (C-Quant)

Measurements	First	Second	Third	Mean
FSCM 1	1.50	1.53	1.46	1.50
FSCM 2	1.66	1.69	1.74	1.70
FSCM 3	1.62	1.65	1.63	1.63
Mean				1.62

Suturing

Suturing tests with the two FSCMs revealed in both methods no tearing at the suture points and the knots could be buried. On bringing in the suture needle, confined cracking occurred. Micro-shearing at the suture points of the FSCM (and not of the human cornea) was observed on tightening some of the knots.

Short-Term Biocompatibility

To determine the scaffold's biocompatibility, FSCMs were placed in the cornea as ALKs, placed intracorneally (intralamellar pockets) and placed subconjunctivally.

One week following transplantation, all six rats that underwent ALK had a clear and quiet cornea, with minimal neovascularization in maximally one quadrant. After 2 weeks, the scaffolds remained in place, while neovascularization was reaching the FSCM. In five cases, a mild haze at the edge of the FSCM, not obscuring the pupil, was observed, and in the sixth animal, the haze partially obscured the pupil. Sutures started to loosen slightly in all cases. At the 3-week end point, the neovascularization extended underneath the FSCM with vessels aimed at the sutures, but not penetrating the FSCM. The haze increased slightly, not obscuring the pupil. Gradual loosening of the sutures resulted in loss of the FSCM in two cases at day 18 and 21, as the rats were allowed to move freely and touch their eyes. These cases were excluded from further analysis.

During follow-up, the eyes were stained with fluorescein to analyze epithelialization. In the ALK group, the total area of the FSCM stained positively, indicating the absence of reepithelialization of the FSCM surface after 3 weeks.

Implantation in an intralamellar pocket was performed in six rats. The results were less uniform than found for the ALK implants. After 1 week, two cases showed no haze, two cases exhibited a mild haze that did not obscure the pupil, and the remaining two rats showed a moderate haze, partially obscuring the pupil. In four cases, blood vessels approached the sutures located near the limbus. All sutures were removed after 1 week. After 2 weeks, superficial vessels reached the FSCM. The amount of haze remained stable, while some melting of the anterior lamella in front of the FSCM was noticed in five animals. Two animals were killed at 13 and 19 days postimplantation due to too much weight loss. When the 3-week end point was reached, blood vessels reached the FSCM in the remaining four animals, with some vessels starting to invade the over-or underlying stroma. Vessels did not invade the matrix and were less dense than in the ALK group.

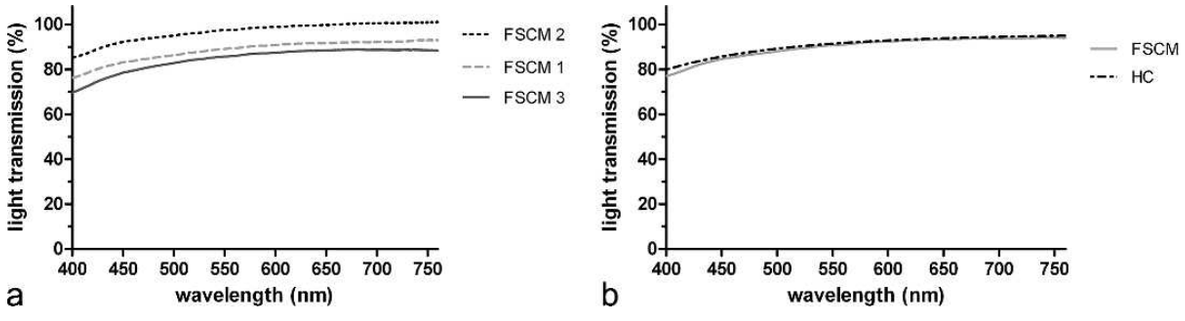

Figure 4. Direct light-transmission curves for the visible spectrum of three FSCMs (a), and the mean direct light transmission of the FSCM compared with the total light transmission of the human cornea (b). HC, human cornea.

After 3 weeks, the corneal haziness had slightly increased in three animals, ranging from a mild haze to an opaque area totally obscuring the pupil. In one case, however, the neovascularization had diminished and remained present only in the limbal area and a moderate corneal haze was observed. Corneal fluorescein staining revealed melted areas of the anterior lamella of variable size in all four remaining cases.

The six rats receiving an SC implant showed slight SC swelling postoperatively at the implantation site. The swelling disappeared after 2 weeks in four rats and lasted until the end point in two rats. Local conjunctival hyperemia was present at a low degree until the end point. Sutures were not removed, as within 1 week the sutures were overgrown by the conjunctiva and removal without damaging the surrounding area proved to be difficult.

Overall

Except for the two cases mentioned, all implanted animals were in good health, kept their weight, and showed no symptoms of any ocular infection or noticeable irritation. The FSCM flattened the recipient rat cornea due to a curvature disassociation. End point neovascularization and opacification (Figs. 5a, 5b) did not differ significantly ($P = 0.46$ and $P = 0.19$, respectively) between the ALK and IL groups. Typical cases of each implantation model are depicted in Figure 6. An overview of in vivo and histological characteristics is given in Table 2.

Histopathological Evaluation

To visualize the clinically observed changes with histology, sections of the four scaffold-containing corneas in the ALK group were cut, stained with HE, and compared with the control lateral eye (Figs. 7a, 7b). HE staining showed mild corneal edema with blood vessels and infiltrating leukocytes in the stroma around the sutures and at a lower degree under the FSCM (Figs. 7c, 7d). A few leukocytes were seen within the FSCM. The leukocytes showed a typically chronic inflammatory reaction with mainly macrophages and lymphocytes and some neutrophils. Epithelial downgrowth occurred in all cases at the border of the FSCM. At the location of the sutures and at the border of the FSCM, the epithelium showed hyperplasia and metaplasia.

HE staining of the four cases of the IL group showed infiltration of immune cells, which consisted of the typical mixture with macrophages, lymphocytes, and some neutrophils. A few leukocytes were found within the FSCM (Fig. 7e). Local edema, metaplasia, and necrotic cells of the epithelium overlying the FSCM were observed in three of the four cases (Figs. 7e, 7f). However, one of the implants had no leukocytes or blood vessels infiltrating the stroma (Fig. 7f) and the other only a few (not shown). This corresponded with the in vivo observations (Figs. 6f–j). Infiltration of immune cells was not significantly different between the ALK and IL groups ($P = 0.57$) (Fig. 8).

The SC implants were completely surrounded by leukocytes in all six cases, with several leukocytes invading the FSCM. The leukocyte infiltration was limited to the adjacent surrounding tissue, again

Table 2. Overview of in vivo and histological characteristics

	Follow-Up (days)	FSCM lost	Corneal vessel score d 21	Opacity score d 21	Excluded on histology	Corneal epith. atrophy	Corneal epith. hyperplasty	Corneal epith. Meta-plasia	Corneal epith. down growth	Leukocyte infiltration
ALK 1	21	N	2	1	N	-	+++	+	Y	++
ALK 2	21	N	3	3	N	+	++	+	Y	+++
ALK 3	21	N	3	1	N	+	++	+	Y	+
ALK 4	21	Y	3	0	N	N/A	N/A	N/A	N/A	N/A
ALK 5	21	N	3	2	N	+	++	++	Y	++
ALK 6	18	Y	-	-	Y	N/A	N/A	N/A	N/A	N/A
IL 1	13	Y	-	-	Y	N/A	N/A	N/A	N/A	N/A
IL 2	13	N	-	-	Y	N/A	N/A	N/A	N/A	N/A
IL 3	21	N	3	2	N	+	++	+	N	++
IL 4	21	N	2	2	N	++	++	+	N	-/+
IL 5	21	N	3	3	N	+	+	+	N	++
IL 6	21	N	1	2	N	++	+	++	N	-
SC 1	21	N	0	0	N	N/A	N/A	N/A	N/A	+++
SC 2	21	N	0	0	N	N/A	N/A	N/A	N/A	+++
SC 3	21	N	0	0	N	N/A	N/A	N/A	N/A	+++
SC 4	21	N	0	0	N	N/A	N/A	N/A	N/A	+++
SC 5	21	N	0	0	N	N/A	N/A	N/A	N/A	+++
SC 6	21	N	0	0	N	N/A	N/A	N/A	N/A	+++

Epith. = epihtelial; d=day; Y = yes; N = no; N/A = not applicable.

Corneal Vessel score: 0 = none; 1 = at the limbus; 2 = reaching sutures / FSCM; 3 = under FSCM; 4 = in FSCM; 5 = whole FSCM.

Opacity score: 0 = transparent; 1 = mild haze, not obscuring the pupil; 2 = moderate haze partially obscuring pupil; 3 = opaque area totally obscuring pupil.

representing chronic inflammation. The SC group showed significantly more infiltration than the ALK group (P = 0.04) and the IL group (P = 0.02) (Fig. 8). The tissue surrounding the implant showed edema (Figs. 7g, 7h). Compared with nonoperated control eyes, more vessels were present.

Discussion

Pattern and Light Scatter and Transmission

The composed phase-contrast image of the FSCM shows that the pattern that contains the micro channels covers only a quarter and not the full surface. This is in accordance with the natural pattern of the so-called ctenoid fish scales.[35] Nevertheless, this nonhomogeneity of the pattern does not prevent corneal stromal cells from populating the scaffold's surface, and is perhaps even stimulating cell spread.[25] The circular running ridges had a height and width much larger than the visible wavelengths, thereby provoking light scatter. This is in accordance with the observations that the pattern was clearly visible under the phase-contrast microscope. The latter visualizes differences in refraction, which may cause light scattering.

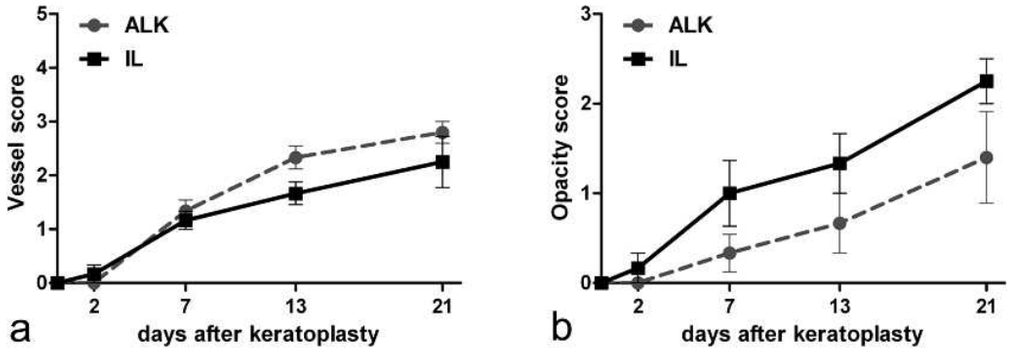

Figure 5. Scoring of corneal neovascularization (a) and opacification (b) at different time points during follow-up of the ALK and IL groups. Vessel score and opacity score did not differ significantly at the 21-day end point (respectively P = 0.46 and P = 0.19; $\chi 2$ trend test). Greatest difference of vessel score between the two groups at day 13 was not significant either (P = 0.14). Error bars represent SEM. Vessel score: 0 = none; 1 = at limbus; 2 = reaching sutures/FSCM; 3 = under FSCM; 4 = entering FSCM; 5 = whole FSCM. Opacity score: 0 = transparent; 1 = mild haze, not obscuring the pupil; 2 = moderate haze partially obscuring the pupil; 3 = opaque area totally obscuring the pupil.

The FSCM had a forward light scattering of log(s) 1.62, comparable to the amount of scattering caused by early cataract.30,36 The direct light transmission was 90%.

Even though this value reflects the direct transmission and not the total light transmission, which includes forward-scattered light as well, it is very close to the total light transmission of 91% found for the human cornea.

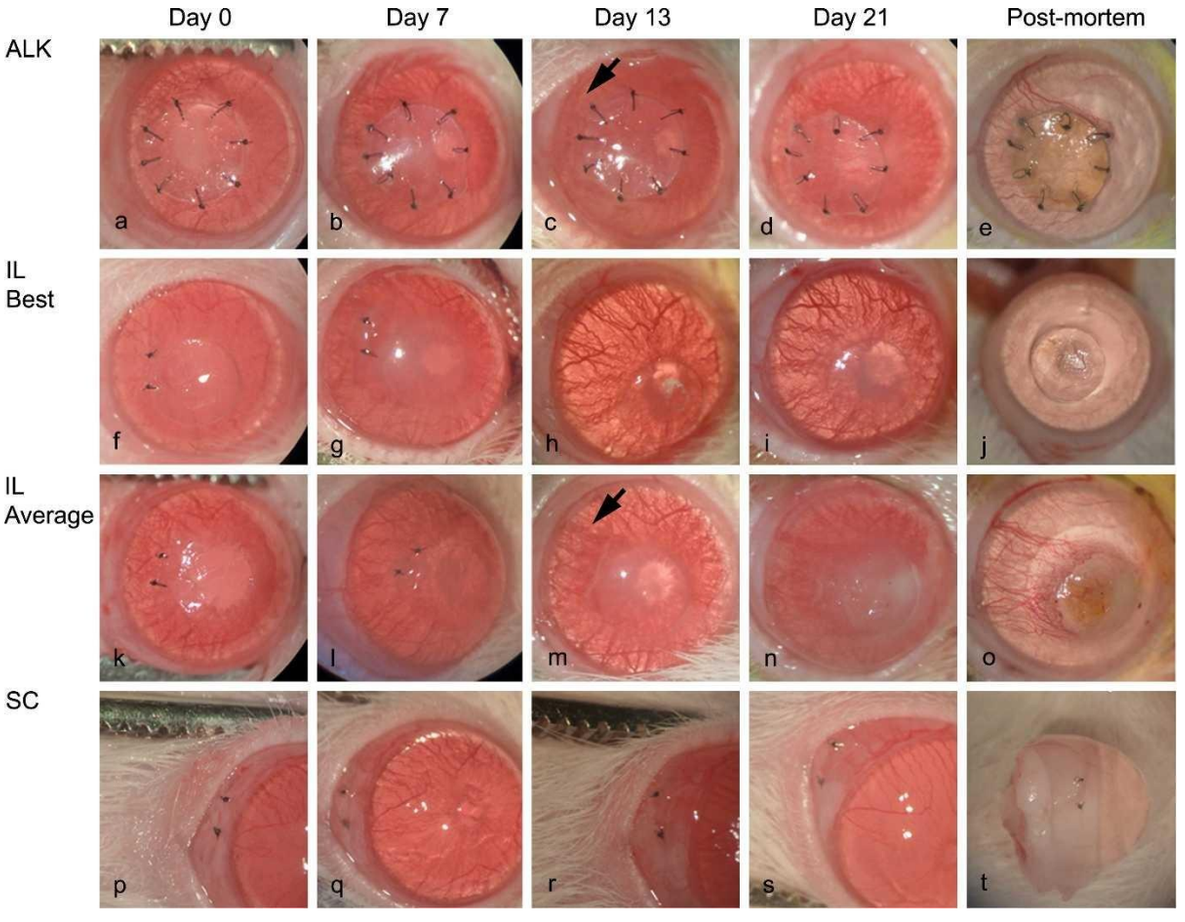

Figure 6. Typical cases of each implantation model are shown. The IL group has been separated in best result and average result, due to high variability. All groups showed good transparency of the corneal implants on microscopic examination directly postoperatively (first column: [a, f, k, p]) and up to 7 days (second column: [b, g, l, q]). Neovascularization and opacification were visible at 13 days (third column: [c, h, m, r]) and gradually increased until day 21 (fourth column: [d, i, n, s]). Neovascularization is seen even more clearly postmortem (last column: [e, j, o, t]).

The light-transmission curves of the FSCM and human cornea are also very similar in shape, indicating corresponding values of Rayleigh scatter (i.e., light scatter caused by particles much smaller than the wavelength of light such as molecules). The displacement along the y-axis of the light-transmission curves for the different FSCMs can be attributed to a wavelength-independent scatter, plausibly caused by the micro pattern that roughens the surface of the FSCM with ridges larger in size than the wavelength of visible light. Although special care was taken for optimum position, it could not be prohibited that each FSCM was slightly differently positioned relative to the spectrometer and therefore caused different amounts of scatter. Removal of the micro pattern from the FSCM surface will improve light transmission and especially light-scatter values, which in turn will result in better visual acuity when implanted.

Suturing

The suturing test demonstrated that the FSCM can be sutured successfully into the cornea, when handled with care. The scaffold was brisker and had less mechanical strength than the human cornea (or rat cornea), indicated by the confined cracking and shearing. Increasing the elasticity and strength likely will improve the ease of handling and this may be needed to make the FSCM applicable for full-thickness transplantation in humans.

In Vivo Biocompatibility

The rat keratoplasty model has been used to study corneal transplantation for more than 25 years.[27] Our findings of this first in vivo study with the FSCM correspond to the results seen with allogeneic corneal transplantations in rats.[37–39] All corneal implants showed good acceptance up to 1 week postoperatively with neovascularization limited to one quadrant of the limbal rim, no opacity at all in the ALK group, and mild opacity in the IL group. After 3 weeks, neovascularization had increased until it reached the FSCM and sutures, but vessels failed to penetrate the FSCM. The opacity gradually increased to a mild haze (stage 1) in the ALK group and a moderate haze (stage 2) in the IL group. Total opacification (stage 3) of the FSCM, indicative for a complete rejection, was observed in only one animal of the IL group. It is important to note that in humans with a nonvascularized cornea, a non-HLA–matched allogeneic corneal transplantation has a high acceptance rate, whereas in rats, this situation leads to rejection after 5 to 15 days.[37–39] A rejected graft is recognized by complete corneal opacification and infiltrating T cells on histology. The main finding of this study on the short-term biocompatibility and handling of the FSCM to obtain a first impression of its use as a replacement of human donor material, is that the FSCM may have potential for corneal applications in humans.

The local abnormalities that occurred in the corneal epithelium are largely explained by mechanical irritation induced by the FSCM. The edges were standing out due to a relative mismatch in curve and thickness and insufficient elasticity. This led to compression of the overlaying stroma and epithelium and created a tear meniscus in the IL group. These characteristics also explain the development of

epithelial downgrowth that occurred in the ALK group. The dense structure and thickness of the FSCM may have put pressure on the remaining corneal tissue in the IL group, prohibiting adequate flow of nutrients from the anterior chamber toward the overlaying anterior cornea. This mechanism, together with a too shallow implantation, is thought to be responsible for the observed melting. Histology confirmed that the cases with lesser melting were indeed implanted at a greater depth.

The amount of neovascularization was comparable to that seen in allogeneic corneal transplantations in rats[38,40] and provoked by the sutures.[41] This explains the higher degree of neovascularization in the ALK group, in which more sutures were used and not removed, in contrast to the IL group, in which two sutures were used and removed after 2 weeks. In both groups, vessels failed to invade the FSCM, perhaps due to the fibril density of the FSCM or by yet to be identified antiangiogenic properties.

Corneal opacification, which indicates swelling or an ongoing immune reaction, reached levels compatible with a complete rejection in 2 of the 12 corneal implants. The observed opacification may be due to the mentioned mechanical irritation, which leads to corneal damage and subsequent leukocyte infiltration.

The infiltration of leukocytes was mild to moderate and consisted predominantly of macrophages, lymphocytes, and some neutrophils, indicative of a chronic immune reaction. The leukocytic infiltration was primarily aimed at the sutures in the ALK group and surrounded the scaffold in the SC group and partly in the IL group. The SC group showed the presence of fibroblasts aligned around the scaffold, similar to a mild foreign body reaction.[42] It is unlikely that this infiltration and swelling is caused by pathogens present on the FSCM prior to implantation, as FSCMs from the same batch were tested and found to be negative for bacteria and fungi. Furthermore, a more severe reaction is expected in case of infection. Type 1 collagen of fish is similar to that of humans[43] and reconstituted tilapia collagen sponges caused only rare inflammatory responses.[26] Still, the collagen of the FSCM or residues of chemicals used for decellularization and decalcification could have provoked the immune response. A strong argument to support the notion that the inflammation was due to surgical trauma and mechanical irritation is the complete absence of inflammation in one case. If the inflammation had been due to a heterologous protein, one would have expected an immune response in all cases. The gradual tearing of the sutures through the FSCM, as noticed after 2 weeks, was aggravated by the outstanding edges and additional external mechanical forces caused by blinking and eye movements. Placing a contact lens or increasing the elasticity or curvature of the FSCM may offer a pathway to prevent this.[44] The increased cellular infiltration and swelling on the histology of the SC group is well explained by the less immune privilege of the conjunctiva compared with the cornea.

Other collagen matrices used for corneal regeneration, such as the biosynthetic artificial cornea,[19] are already being used in clinical studies, following a decennium of preclinical research.[45] The novelty and advantage of the FSCMs are its wide availability, its low cost, and simple manufacturing process. However, we are at an early phase in our research, and working on overcoming technical challenges so as to develop a useful prototype.

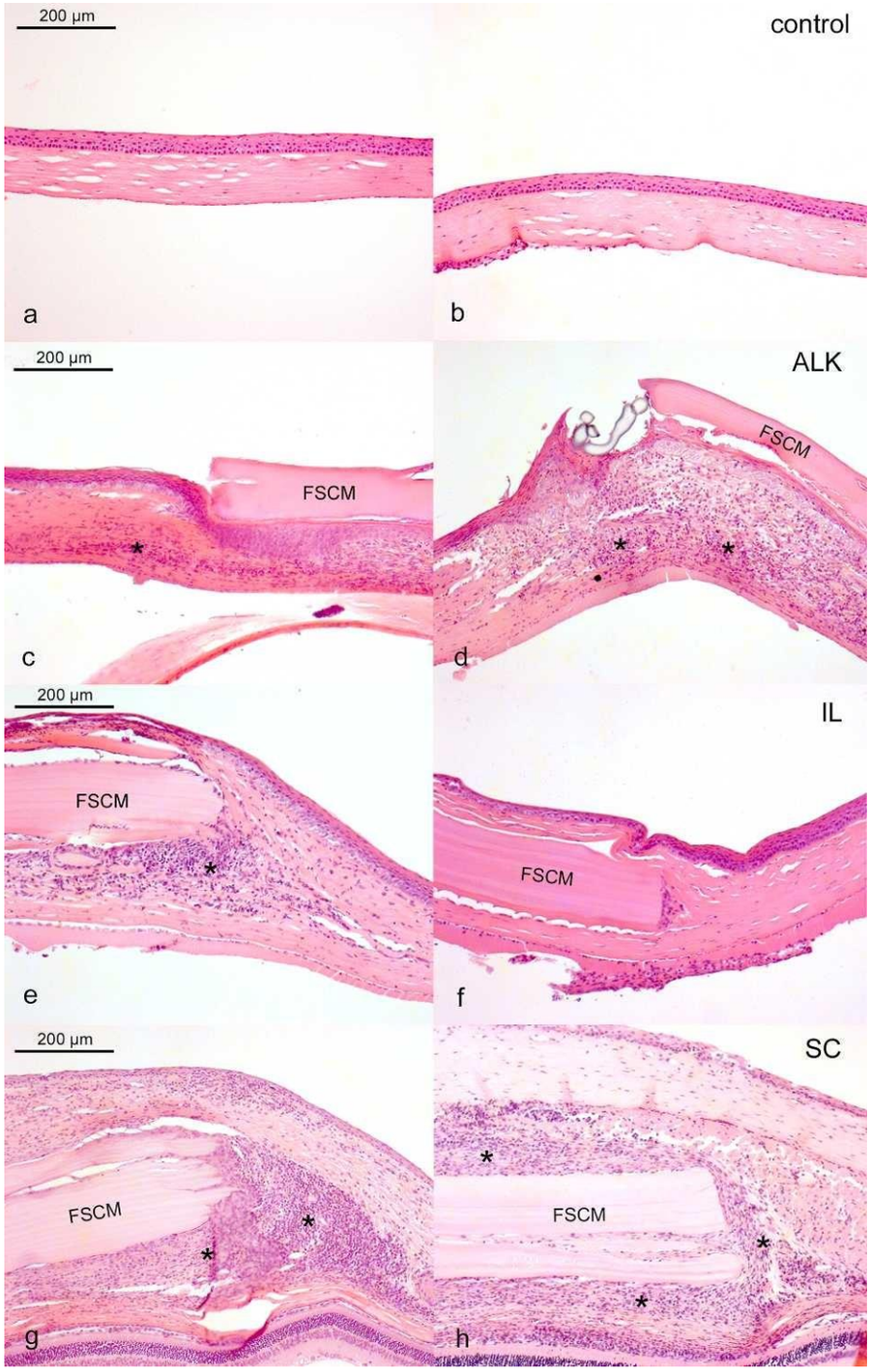

Figure 7. HE stainings. Examples of control corneas are shown (a, b). The ALK group had mild to moderate infiltrationof leukocytes, epithelial hyper- and metaplasia, and epithelial downgrowth due to mismatch in shape (c, d). The IL group had a mild to moderate infiltration in two cases (e) and almost no infiltration in two other cases (f); all IL cases showed local atrophy and metaplasia of the epithelium (e, f). The SC group showed moderate infiltration (g, h), with fibrous capsule formation and some leukocytes infiltrating between the layers (h). *Leukocyte infiltration and concomitant edema.

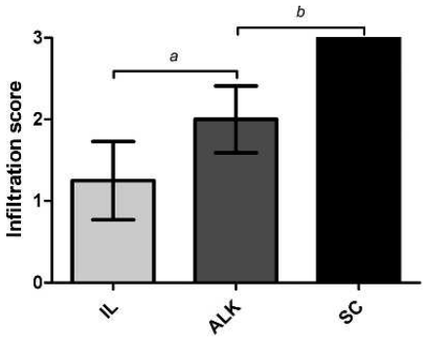

Figure 8. Semiquantitative scoring of stroma infiltrating leukocytes (0 = none; 1 =+; 2 = ++ 3 = +++). Error bars represent SEM. Differences were tested with $\chi 2$ test ([a]: P = 0.57; [b]: P = 0.04).

Conclusion

This systemic study of the physical and biomedical short-term effects of the FSCM as corneal replacement, demonstrates its potential for future use. The availability of an easy obtainable and biocompatible FSCM can have a high impact on decreasing the shortage of donor corneas and make lifelong use of immunosuppressive medication redundant. The potential of this FSCM is demonstrated for the first time by an adequate light transmission, reasonable light-scattering values, and ability to be used in keratoplasty. Future studies with a curved and thinner FSCM are necessary to prevent mechanical irritation and increase the understanding of its immunogenicity. Long-term in vivo studies and studies on modified FSCMs, for instance with the top pattern removed, are needed to develop and optimize this readily available collagen matrix as an artificial cornea.

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CHAPTER 3

Biocompatibility of a fish scale-derived artificial cornea: Cytotoxicity, cellular adhesion and phenotype, and in vivo immunogenicity

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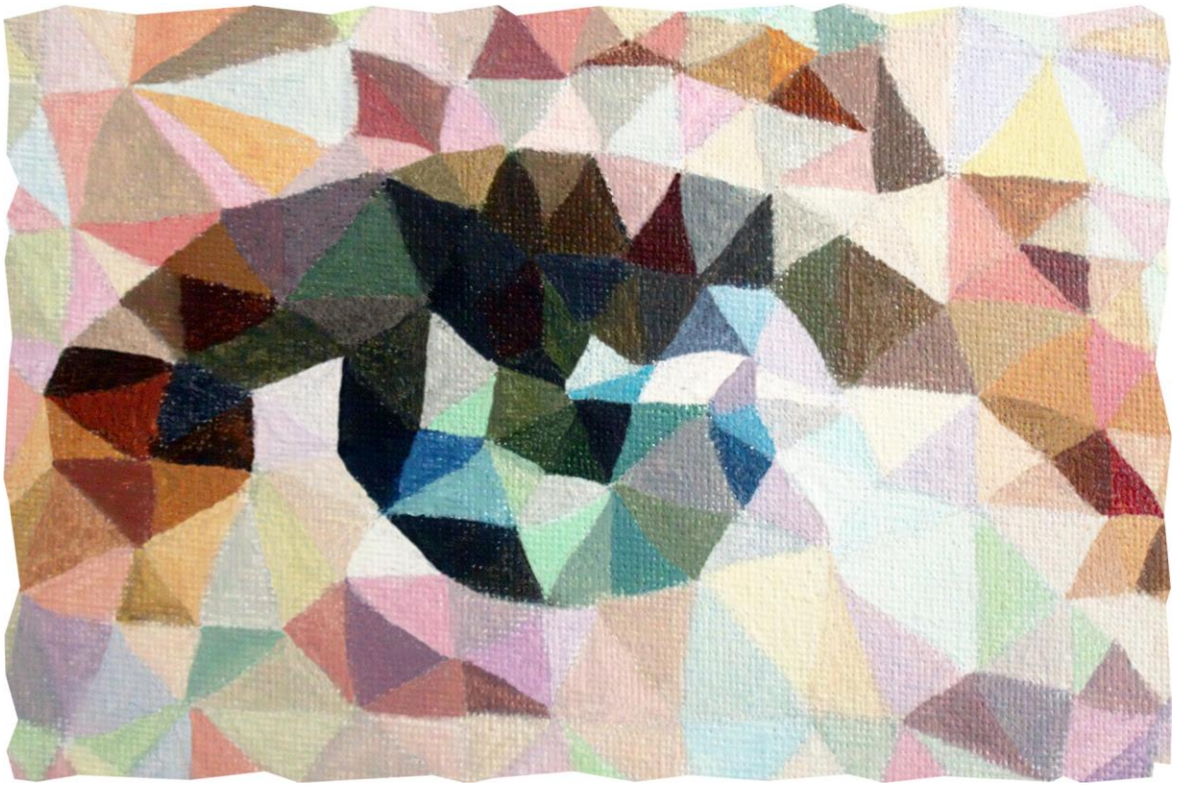

Abstract

Purpose: To determine whether a fish scale-derived collagen matrix (FSCM) meets the basic criteria to serve as an artificial cornea, as determined with in vitro and in vivo tests.

Methods: Primary corneal epithelial and stromal cells were obtained from human donor corneas and used to examine the (in)direct cytotoxicity effects of the scaffold. Cytotoxicity was assessed by an MTT assay, while cellular proliferation, corneal cell phenotype and adhesion markers were assessed using an EdU-assay and immunofluorescence. For in vivo-testing, FSCMs were implanted subcutaneously in rats. Ologen® Collagen Matrices were used as controls. A second implant was implanted as an immunological challenge. The FSCM was implanted in a corneal pocket of seven New Zealand White rabbits, and compared to sham surgery.

Results: The FSCM was used as a scaffold to grow corneal epithelial and stromal cells, and displayed no cytotoxicity to these cells. Corneal epithelial cells displayed their normal phenotypical markers (CK3/12 and E-cadherin), as well as cell-matrix adhesion molecules: integrin-a6 and b4, laminin 332, and hemi-desmosomes. Corneal stromal cells similarly expressed adhesion molecules (integrin-a6 and b1). A subcutaneous implant of the FSCM in rats did not induce inflammation or sensitization; the response was comparable to the response against the Ologen®Collagen Matrix. Implantation of the FSCM in a corneal stromal pocket in rabbits led to a transparent cornea, healthy epithelium, and, on histology, hardly any infiltrating immune cells.

Conclusion: The FSCM allows excellent cell growth, is not immunogenic and is well-tolerated in the cornea, and thus meets the basic criteria to serve as a scaffold to reconstitute the cornea.

Introduction

Of the 39 million people worldwide who suffer from corneal blindness, approximately 12% are affected bilaterally, with the developing countries having the highest percentages of corneal blindness.[1-3] Corneal blindness may be caused by corneal opacities, trachoma, and childhood blindness.[3] There are multiple barriers that prevent restoration of vision in these patients through corneal transplantation, and these problems are especially common in developing countries: lack of access to drugs such as steroids and antibiotics, and lack of trained surgeons, but the most important one is lack of corneal donor tissue and the availability of those corneas at an affordable price.[3,4] Even in the developed countries the cost of a donor cornea remains high, for example the cost of a donor cornea in the United States is approximately 3000 US dollars.[5]

The shortage of donor corneas may be reduced by using keratoprostheses as artificial corneas, as these keratoprostheses, such as the Boston Keratoprosthesis (B-Kpro) and Osteo-Odonto-Keratoprosthesis (OOKP), are viable solutions.[6,7] However, due to potential complications and high surgical demands, such implants are currently only used in high risk patients. Furthermore, the B-Kpro still has the risk of rejection as it uses a donor cornea, while the OOKP has a complex implantation procedure, and both are rather expensive, although the developers of the B-Kpro are working towards cost reduction.[8] Three-dimensional scaffolds made from biomaterial, mimicking the corneal stroma, may be repopulated by the patient's own corneal cells, and those cells may in turn regenerate corneal tissue.[9-11] The use of biomaterials would reduce the chance of immune rejection. Three-dimensional scaffolds can be fabricated,[10] or harvested in an almost ready state from, for example, porcine corneas,[12] or a porcine cornea may be modified before use.[13]

Different biomaterials can be used and indeed are being explored as the basis for scaffolds.[4,14-17] The human corneal stroma consists primarily of type I collagen, arranged in orthogonal lamellae, which give the cornea its enormous tensile strength.[18] This makes collagen a logical material for use as an artificial corneal scaffold.[15,19] Indeed, one synthesized collagen (recombinant human collagen type III) scaffold has reached the clinical phase I, but a drawback is the complicated fabrication process;[20] another one is achieving enough tensile strength of the construct to allow interrupted or continuous suturing.

Decellularized human corneas can also be regarded as collagen-based scaffolds but use of these scaffolds will not decrease the lack of need for tissues. Decellularized porcine corneal stroma is another option, but this confers a risk of transmitting animal disease.[15] Screening for pathogens may reduce that risk.[15]

Another source for three-dimensional collagen scaffolds are fish scales.[21-23] Scales from the Tilapia fish, farmed under controlled conditions,[24] are composed of collagen type I, arranged in a similar way as in human corneas.[25] Such scaffolds may offer a cheap alternative and facilitate corneal regeneration.

Previous studies showed that a collagen scaffold, made of the scales of the Tilapia fish (Oreochromis niloticas) by decellularization and decalcification, facilitates repopulation by rabbit corneal stromal cells and has a high oxygen permeability.[23] Our previous experiments showed that this scaffold has adequate light transmission values and is suitable for use in keratoplasty, using intermittent sutures.[26]

As we hypothesized that the fish scale-derived collagen matrix (FSCM) can be used as an implant for corneal reconstruction, we set out to perform an in depth investigation into the suitability of this implant for corneal reconstruction. We measured the tensile strength and glucose permeability of the FSCM-derived scaffolds, cultured primary human corneal epithelial and stromal cells together with the scaffold to assess the scaffold's cytotoxicity, and determined its effect on the proliferation, phenotype and adhesion of corneal cells in vitro; finally, we studied the body's response upon subcutaneous and corneal intrastromal placement of the FSCM in rats and rabbits, respectively.

Methods

Fish-scale derived collagen matrix

The collagen matrices were prepared from the scales of Tilapia (Oreochromis Niloticas), bred under controlled conditions as previously described.[23,24,26] Decellularized, decalcified and gamma-irradiated FSCM were provided by Aeon Astron Europe BV (Leiden, the Netherlands) and stored in sterilized phosphate-buffered saline (PBS) containing 1% penicillin/streptomycin (15140-122; Gibco by Life Technologies, Bleiswijk, the Netherlands) at 4° Celsius until use. The matrices were 8.0 ± 1.0 mm in diameter, 0.25 ± 0.05 mm in thickness, and had no curvature, except the ones used for the rabbit experiment.

Tensile strength and glucose permeability

The tensile strength of the matrices was determined using a universal testing machine (Instron 3365, UK) with a gauge length of 10 mm. The test was performed at a crosshead speed of 6 mm/min at room temperature. Measurements were performed on four independent samples.

Glucose permeability was assessed with the USP <1724> Vertical Diffusion Cell Test using the Franz cell system. Matrices (n = 4) were fixed between a donor chamber containing 50 mg/ml glucose solution and a receptor chamber with distilled water. After 1, 6, and18 h, water from the receptor chamber was removed for glucose measurement, for which a glucose assay kit was used (Cat no. GAGO20-1KT; SigmaeAldrich, Diegem, Belgium).

Primary human corneal cells

Human cadaveric eyes were obtained from the EuroCorneabank, Beverwijk, the Netherlands. Corneal epithelial cells were harvested by cutting the cornea into four equal quadrants, washing the corneal pieces three times in PBS (phosphate-buffered saline) and incubating the tissue overnight at 4 °C in 2.4 U/ml dispase II (04 942078 001; Roche Applied Science, Mannheim, Germany) in PBS. The corneal

epithelium was manually separated as a sheet from the underlying tissue by gentle use of forceps and a spatula, centrifuged (5 min at 1000 rpm), and incubated in 80 ml TrypLE (TrypLE Select 126050-10; Life Technologies Europe BV, Bleiswijk, the Netherlands) for 10-15 min at 37 °C to create a single cell suspension, which was cultured in CnT-20 medium (CnT-20; Bio-connect BV, Huissen, the Netherlands) with 1% penicillin/streptomycin.

The cornea was cut into small pieces of about 1 1 mm to isolate corneal stromal cells. The corneal parts were placed in a 0.1% collagenase type II solution (17101-015; Life Technologies Europe BV) and incubated overnight at 37 °C. The obtained cell solution was put through a 70 mm cell strainer, subsequently centrifuged (5 min at 850 rpm) and cultured in DMEM/HAM F12 medium with stable glutamin (FG4815; Biochrom AG, Berlin, Germany), supplemented with 5% fetal calf serum (758093; Greiner Bio-one GmbH, Frickenhausen, Germany) and 1% penicillin/streptomycin.

Cytotoxicity and proliferation

Cytotoxicity was analyzed using an MTT viability assay, where epithelial and stromal cells were directly and indirectly exposed to the FSCM (P12261201/P12041201) for 24 h. In order to determine cytotoxicity through direct cell contact, the cells were seeded and the scaffold was added to the culture. To assess any indirect cytotoxicity, the cells were cultured in the presence of FSCM extract (cell culture medium that had been incubated with the scaffold for 24 h at 37 °C) and a dilution series of the extract (1:2 and 1:4). As a positive control, cells were treated with 1% sodium dodecyl sulfate, while untreated cells served as a negative control. For the MTT assay, 1 mg/ml 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) (M2128-5G, lot: 046K5304; SigmaeAldrich, Diegem, Belgium) was added to the cell cultures and the cells were incubated for 2 h at 37 °C. Metabolically active cells reduce MTT to formazan and after formazan extraction using isopropanol, the optical density was measured using a spectrophotometer (at 570 nm). This assay was performed in triplicate.

To assess proliferation, the two-donor mixes of epithelial (40,000 cells) and two-donor mixes of stromal cells (25,000 cells) were seeded onto the smooth side of the FSCM (P12261201) and on plastic discs, which served as controls. Experiments were carried out in duplicates. After 24, 48, and 72 h, cells were incubated with1% of 5-ethynyl-2'-deoxyuridine (EdU) (Click-It® EdU Alexa Fluor® 594 Imaging Kit; C10339; Life Technologies Europe BV) for 16 h, and, after being washed with PBS, cells were fixed with 4% form-aldehyde in PBS, and permeabilized by 0.5% Triton® X-100 EdU was visualized using Alexa Fluor® 594. DAPI counterstaining served to identify cell nuclei. EdU-positive cells, indicating actively dividing cells, were manually counted by two different observers in three randomly-selected areas on the FSCM, using a confocal microscope.

Immunofluorescence, SEM and TEM

Corneal epithelial cells were seeded on the patterned side of four FSCMs (15,000-50,000 cells/FSCM), and on the smooth side of two FSCMs (P06271201), and cultured until sub-confluence (11-12 days),

at which time the FSCMs were prepared for whole mount immunofluorescence to assess cell phenotype and adhesion. For this, the scaffolds were fixed in 4% formaldehyde, and antibodies were applied against CK3/12 (1:50; bs-2369R-Cy7; Bio-Connect BV, Huissen, The Netherlands), a marker of differentiated corneal epithelium, and the cell-matrix adhesion molecules integrin α6 (1:1000; ab20142; Abcam; Cambridge, UK) and β4 (1:1000; ab110167; Abcam; Cambridge, UK). After incubation with fluorochrome-labeled secondary antibodies, counterstaining was performed with DAPI (cell nuclei) and Alexa Fluor 488 Phalloidin (F-actin filaments) (1:100; A12379; Life Technologies Europe BV; Bleiswijk, the Netherlands).

The experiments with the corneal epithelial cells were repeated at another location (University of Antwerp, Antwerp, Belgium) using corneal limbal explants from one human donor cornea. Each limbal explant was placed on top of four different non-curved FSCMs (P12261201), and additionally on top of two dome-shaped FSCMs (P08081304; diameter curvature 7.4 ± 0.2 mm) and cultured in Corneal Epithelial Culture Medium[27] (supplemented with collagen and fibronectin) for 22 days for immunofluorescence and 20 days for analysis with Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM). Whole mount immunofluorescence was performed on the four non-curved FSCMs, that were cut in halves, for CK3/12 (1:100; ab68260; Abcam, Cambridge, UK), E-cadherin (1:400; ab53033), integrin α6 (1:1000; ab20142), laminin-332 (1:25; ab11575; Abcam; Cambridge, UK), and additionally with ΔN P63 (1:300; sc-8609; Santa Cruz Biotechnology; CA, USA), a determinant for stemness. Corneal stromal cells were also cultured on the smooth side of FSCMs (P12261201), coated with and without rat tail collagen type I. The cells were stained for whole mount immunofluorescence with antibodies against the transmembrane receptors integrin-α6 (1:1000; ab20142) and integrin-β1 (1:50; ab52971; Abcam, Cambridge, UK), involved in cell-matrix adhesion, and counterstained for F-actin (Alexa Fluor 488 Phalloidin; A12379) to depict the cytoskeleton, and with DAPI for cell nuclei.

Post-modifications

FSCMs (P04201201)[23,24,26] were treated with femto second laser to create FSCMs with vertical non-penetrating pores only, or in combination with 1x890 mm (HxW) horizontal tunnels. Additionally, two scaffolds were incubated for 3 h at 37 °C in PBS with 4.8 U/ml dispase II to detach the collagen layers. Corneal stromal cells were cultured on these manipulated FSCMs (200,000 cells/FSCM). When complete confluence over the FSCM (2-3 weeks) was obtained, the samples were fixated in 4% formaldehyde and hematoxylin-eosin (HE) staining and immunofluorescence with F-actin and DAPI was performed to visualize the effect on the FSCMs and on the behavior of the cells.

Subcutaneous implantation

Forty male Fischer 344 albino rats (Charles River Laboratories, France), weighing between 280 and 336 g, were used for subcutaneous implantation of the FSCM. Ethical approval was given by the Animal Experiments Committee of the Leiden University Medical Center (Leiden, the Netherlands),

and all animals were treated according to the ARVO statement for use of animals in ophthalmic and vision research. Animals were given at least one week for acclimatization.

Prophylactic pain reduction was started pre-operatively with acetaminophen infusion solution (50 mg/ml) added to the drinking water at 1 mg/ml, which was continued during seven days. The rats were anesthetized with isoflurane and a subcutaneous injection of 50 ml bupivacaine (5 mg/ml) at the site of implantation. A 2 cm long incision was made in the right, high dorsal flank, where a subcutaneous space was created, and the matrix inserted. Twenty animals received the FSCM (P09151101) and twenty other animals another matrix, the Ologen® Collagen Matrix (Aeon Astron Europe, Leiden, The Netherlands; 12 mm in diameter, 1 mm in thickness; porcine collagen type I) as a control; Ologen® Collagen Matrix is used for bleb filtration in trabeculectomy surgery in humans.[28]

All matrices were cut in half and soaked in antibiotics for 5 min (Polyspectran®containing Gramicidine, neomycine and poly-myxine B) prior to insertion. The incision was closed with 6/0 absorbable sutures (Safil REF 1048734, B. Braun Medical BV, Oss, The Netherlands). Seven animals of the twenty with an FSCM, and eight animals of the twenty with an Ologen® implant, additionally underwent sham surgery on the contralateral dorsal flank, which served as a control (n = 15) to determine whether the surgery itself influenced local inflammation in the rat subcutaneous tissue.

At 1, 6, and 11 weeks post implantation, five animals with an FSCM and five with an Ologen® implant were sacrificed. Three weeks after the first implant, a second FSCM was implanted in the contralateral dorsal flank of five animals of the FSCM group and a second Ologen®implant in five animals of the Ologen® group to study immune sensitization; these animals were sacrificed one week after the second implant. After the animals were sacrificed, the subcutaneous tissues were harvested, formalin fixed and embedded in paraffin. Sections were cut and stained with HE. Based on the HE staining, three representative cases were selected out of the five per group per time point, which were additionally stained with antibodies to identify granulocytes (MPO staining; 1:1000; ab45977; Abcam), macrophages (ED-1 staining; 1:300; MAB1435; Millipore BV, Amsterdam, The Netherlands), T cells (CD3 staining; 1:500; MCA772GA; Sanbio BV, Uden, The Netherlands), and immunoglobulins (Polyclonal Rabbit Anti-Rat Immunoglobulins/HRP, mainly IgG; 1:100; P0162; Dako BV, Heverlee, Belgium).

For automatic scoring with a computer program (ImageJ;[29] W.S. Rasband, ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997e2014), three images were made of two subsequent sections per matrix at standard locations (left end, middle, and right end of the matrices), and the percentage positively-staining area per view was calculated for each staining. An independent pathologist graded the amount of immune response as: minimal, mild, moderate, and severe, based on the HE staining.

Intrastromal implantation

Fourteen female New Zealand White rabbits (Charles River Laboratories, France), ranging from 2 to 3 kg, underwent intra-stromal surgery, with seven animals receiving a curved FSCM (P08081304; 120 ± 20 mm in thickness, curvature 7.4 ± 0.2 mm in diameter) in their right eye, while seven other animals received no implant (while undergoing the same surgical procedure), serving as controls. Ethical approval was given by the Animal Experimentation Committee of the University of Utrecht (Utrecht, the Netherlands) where the experiment was conducted, and all animals were treated according to the ARVO statement for use of animals in ophthalmic and vision research. All rabbits underwent at least one week of acclimatization.

Prophylactic painkilling was started preoperatively with a subcutaneous injection of Carprofen (4-5 mg/kg) and this was given postoperatively once daily for six days. The anesthetic regimen consisted of intramuscular injections of Ketanest-S® (ketamine 15 mg/kg/h) and Dexdomitor® (dexmedetomidine 0.125 mg/kg/h), and local administration of one drop oxybuprocaine 0.4% every 15 min.

A corneal incision was made with a precision depth knife set at approximately 80% of the corneal thickness, and the corneal layers were dissected horizontally with a blunt spatula until a pocket was formed of at least 4 mm width and 5 mm length. An FSCM was trephined to 3.8 mm and inserted into this corneal pocket, and the incision was closed with 10/0 nylon sutures. Sutures were removed at 3.5 weeks follow-up.

The eyes were treated postoperatively with tobramycin/dexamethasone eye drops twice daily for eleven days. When symptoms of ocular irritation arose, tobramycin/dexamethason treatment was restarted for all animals, together with artificial tears (Visidic Carbogel; Tramedico BV, Weesp, the Netherlands), until the symptoms subsided, after which the treatment was reduced, but continued until euthanasia after 6 weeks of follow-up.

Eyes were checked with a surgical microscope at 1, 2, 3.5, and 6 weeks for conjunctival hyperemia, corneal haze and neovascularization, and ocular discharge. At 6 weeks, the eyes were additionally observed through a slit-lamp, and the animals were euthanized. The corneas were harvested, fixated in formaldehyde and embedded in paraffin. HE staining was performed as well as an immunohistochemical staining with an antibody against RAM11 (1:25; M063301; Dako BV) to identify macrophages.

Long-term intra-stromal implantation

Two other New Zealand White rabbits were used for long-term follow-up. In these animals a partial depth corneal incision was made to create an intrastromal pocket of 6.5 mm in diameter using a crescent 2.0 mm knife. An FSCM was trephined down to 6 mm in diameter and inserted to the pocket. The incision opening was closed with 10-0 nylon sutures. Postoperatively daily drops of Levofloxacin 0.5% and Prednisolone acetate 1% (Pred Forte) were administred during the first 4

weeks. Follow up was performed at week 1, 2, 3, 4 and 54 with optical coherence tomography (OCT) (iVue100, Optovue Inc.).

Results

Tensile strength and glucose permeability

Four FSCM's were used to measure the tensile strength and glucose permeability. The Young's modulus for tensile strength was calculated from the stress-strain curve and was 11.7 ± 0.92 MPa. The glucose diffusion flux was maximal after 1 h (24.9 ± 6.8 x 10^{-6} cm^2/s). The diffusion flux decreased to 6.0 ± 0.5 and 2. 6 ± 0.3 x 10^{-6} cm^2/s at 6 and 18 h.

Cytotoxicity

Human corneal epithelial and stromal cells were co-cultured with the FSCM to test for any cytotoxic effects using an MTT assay. Cell viability was assessed and compared to unexposed controls (Fig. 1). For the direct contact test, an FSCM was added to the culture, while for the indirect contact test, the cells were cultured in the presence of FSCM extract (cell culture medium that had been incubated with the scaffold for 24 h at 37°C) and a dilution series of the supernatant was tested. Using two FSCM, corneal epithelial and stromal cell viability in the direct contact test was 79.6% and 83.4%, and for the indirect contact test 83.7% and 89.3%. This was higher than the threshold for cytotoxicity, which has been set at 70% according to the ISO-standard (ISO: 10993-5:2009(9)).

Proliferation

Next, we determined whether the FSCM influenced cell proliferation. Human corneal epithelial and stromal cells were seeded on an FSCM and on plastic, and dividing cells were counted after 24, 48, and 72 h of culture using EdU staining. The percentage of actively-dividing (EdU positive) epithelial or stromal cells on the FSCM was similar to the number of EdU-positive epithelial or stromal cells cultured on plastic discs (Fig. 2A and B).

Phenotype and adhesion

After having assessed cytotoxicity and cell proliferation, we determined if there might also be an effect on morphogenesis or on adhesion molecules. Therefore, primary human corneal epithelial cells were cultured on FSCMs and assessed using immunofluorescence for the presence of different markers. The cells proliferated on both the anterior as well as the posterior side of the FSCM, and we observed no difference in cell growth with regard to the different micropatterns present on the FSCM (spokes, ridges, and spikes on the anterior side, and no pattern (smooth) on the posterior side (Fig. 3). The seemingly black, unpopulated areas (Fig. 3B) were out of focus: changing the focus of the confocal microscope showed that these areas were populated as well.

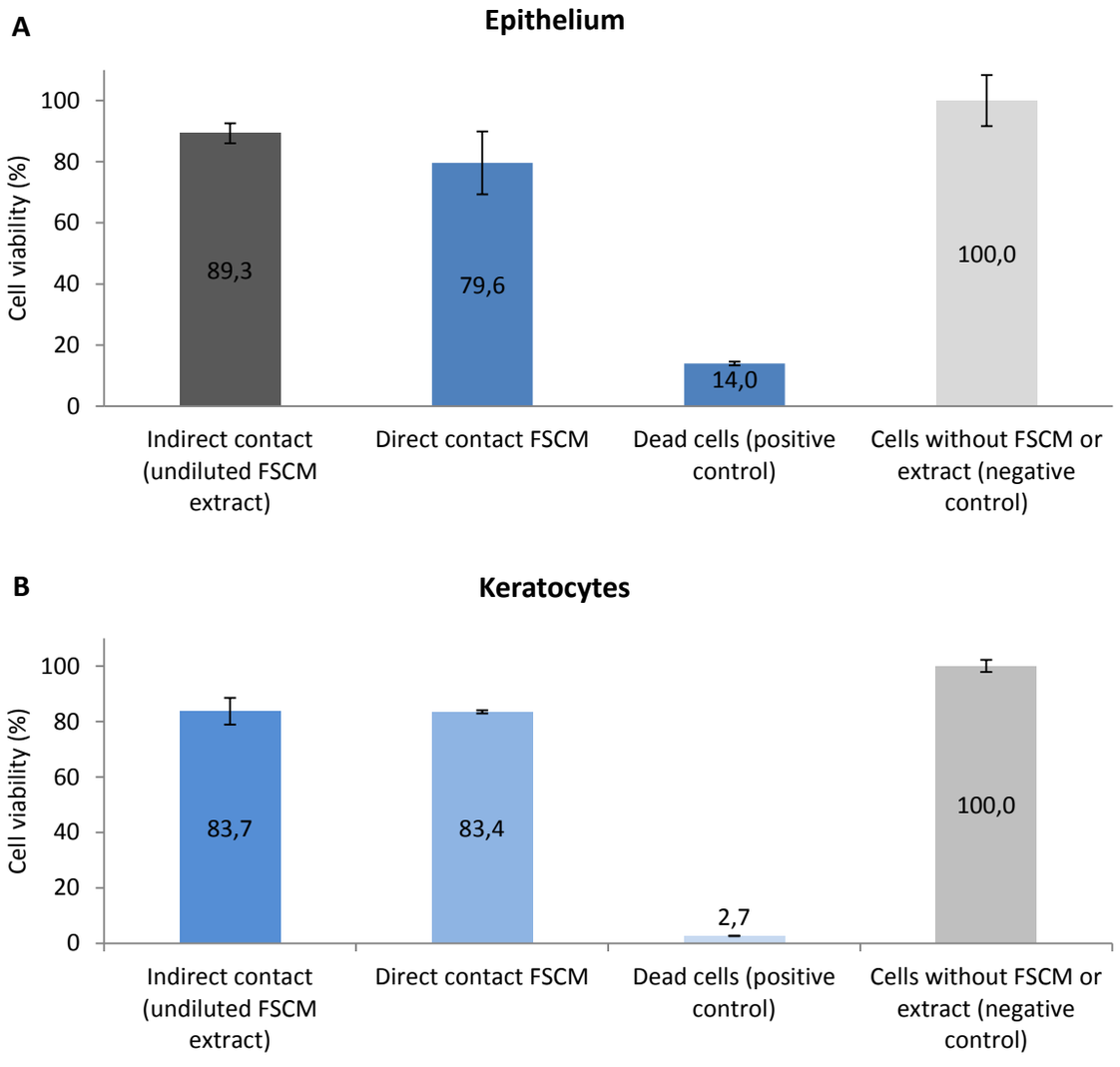

Figure 1. Effect of fish scale-derived collagen matrix (FSCM) on cellular viability. Primary human corneal epithelial cells (A) or primary human stromal cells (B) were cultured in indirect and direct contact with the fish scale-derived collagen matrix (FSCM), and the cytotoxicity was tested using a viability assay (MTT test). As a positive control for minimal MTT staining (toxicity) we used cells killed with SDS.

Primary human corneal epithelial cells were grown until confluence on the FSCM in two different laboratories and assessed for phenotype and adhesion markers with immunofluorescence staining (Fig. 4A). The epithelial cells were CK3/12-positive, and displayed cell-cell adhesion and cell-matrix adhesion molecules (E-cadherin, integrin-α6, integrin-β4, and laminin 332). The primary human stromal cells, the keratocytes, cultured on the FSCM, were positive for integrin-α6, and integrin-β1 (Fig. 4B). No difference in expression was observed when FSCM's were coated with rat tail collagen type 1 (data not shown).

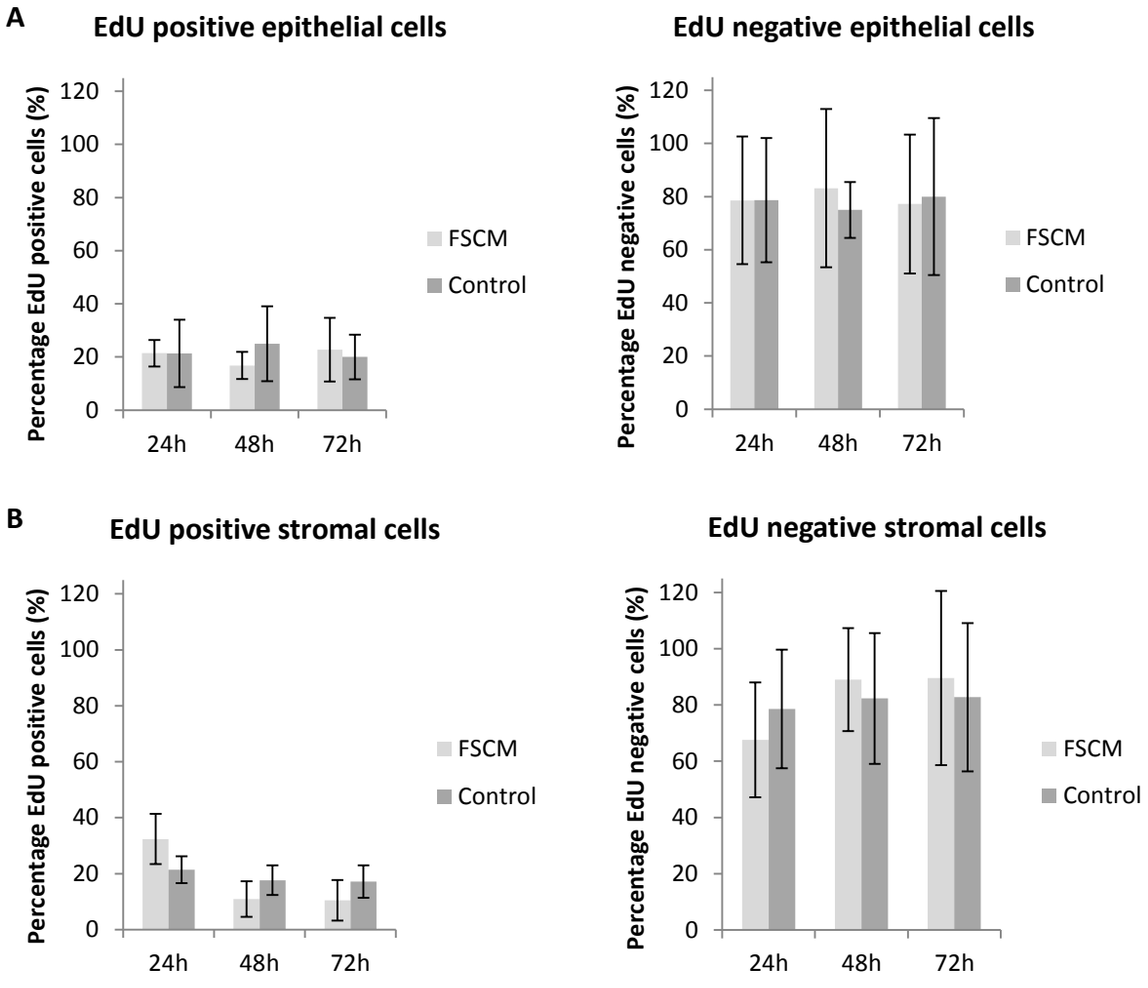

Figure 2. Cell proliferation of human corneal epithelial and stromal cells cultured on FSCM or plastic. Primary human corneal epithelial cells (A) or primary human stromal cells (B), were cultured on the fish scale-derived collagen matrix (FSCM) or on plastic control discs and proliferation was determined in a three-day proliferation assay, in which actively dividing cells were stained with 1% 5-ethynyl-2'-deoxyuridine (EdU). There were no significant differences between the percentage EdU positive and EdU negative epithelial and stromal cells grown on the FSCM and on the control discs (ManneWhitney U test).

The TEM images revealed that stratified primary epithelial cells grown on top of the FSCM developed a stratified layer, which had approximately six cell layers, and that desmosomes were present between the epithelial cells, while hemidesmosomes were seen between the basal epithelial cells and the underlying FSCM (Fig. 4C).

Post-modifications to stimulate cellular ingrowth

We wondered whether it was possible to post-modify the FSCM to stimulate cell ingrowth into the scaffold, and to test this, the FSCM was incubated in dispase and modified by femto-second laser (Fig. 5A). Incubating the FSCM in 2.4 U/ml dispase II for 4 h resulted in a space between the dense collagen layers at the border-area of the matrix (Fig. 5B). We additionally created stromal pores using

a femto-second laser and cultured primary corneal stromal cells on top of this modified FSCM. Confocal immunofluorescence images depicted cells that filled up the pore of the FSCM, as well as the small spaces created between the dense collagen layers (Fig. 5C). When a FSCM was used that had been modified with pores and horizontal tunnels of approximately 1 mm in height, corneal stromal cells were found to protrude their cytoskeleton into those tunnels (Fig. 5D).

Immunogenicity

To determine the immunogenicity of the FSCM, a classical challenge test was performed in rats. The FSCM was implanted subcutaneously in the dorsal flank of rats (n = 20) and the tissue response was compared to a matrix used in human bleb filtration for trabeculectomy (n = 20), as well as to sham surgery (n = 15). The tissue response was homogenous in each group, although some variations in

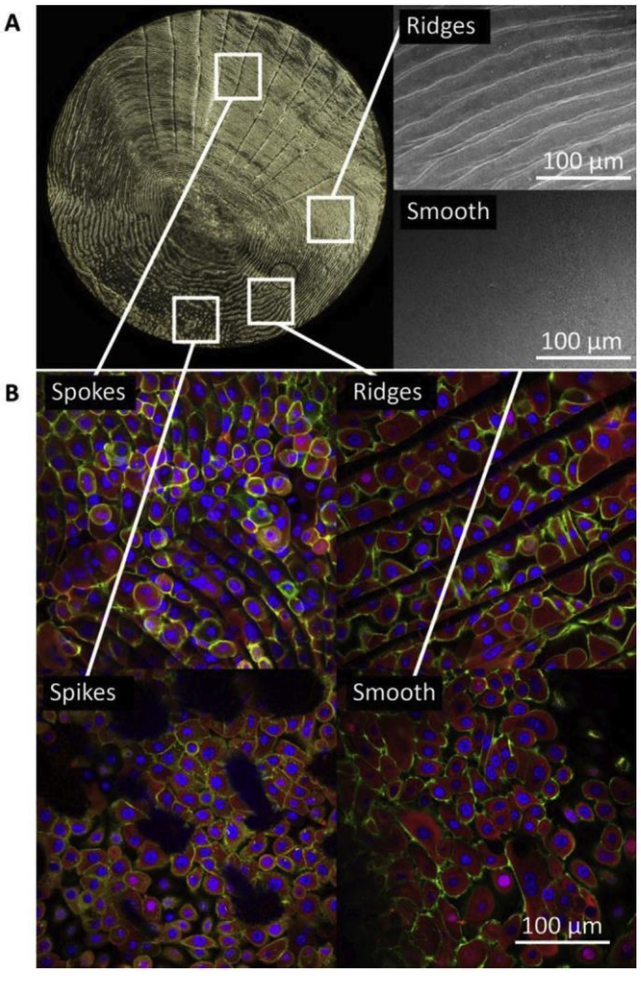

Figure 3. In vitro human corneal epithelial cell growth on the FSCM. The anterior surface of the FSCM has three distinguishable patterns: spokes, ridges, and spikes, as clearly seen with a phase-contrast microscope, while the posterior surface is smooth, as demonstrated by a SEM (A). The primary human corneal epithelial cells grew equally on each surface (B). Blue: DAPI, staining cell nuclei; Green: Alexa Fluor 488 Phalloidin, staining F-actin; Red: CK3/12, staining differentiated corneal epithelium.

amount and composition of infiltrating cells existed and one animal, at the end point, showed fibrous capsule formation. Fig. 6A shows the HE staining of the FSCM and surrounding subcutaneous tissue at 11 weeks.

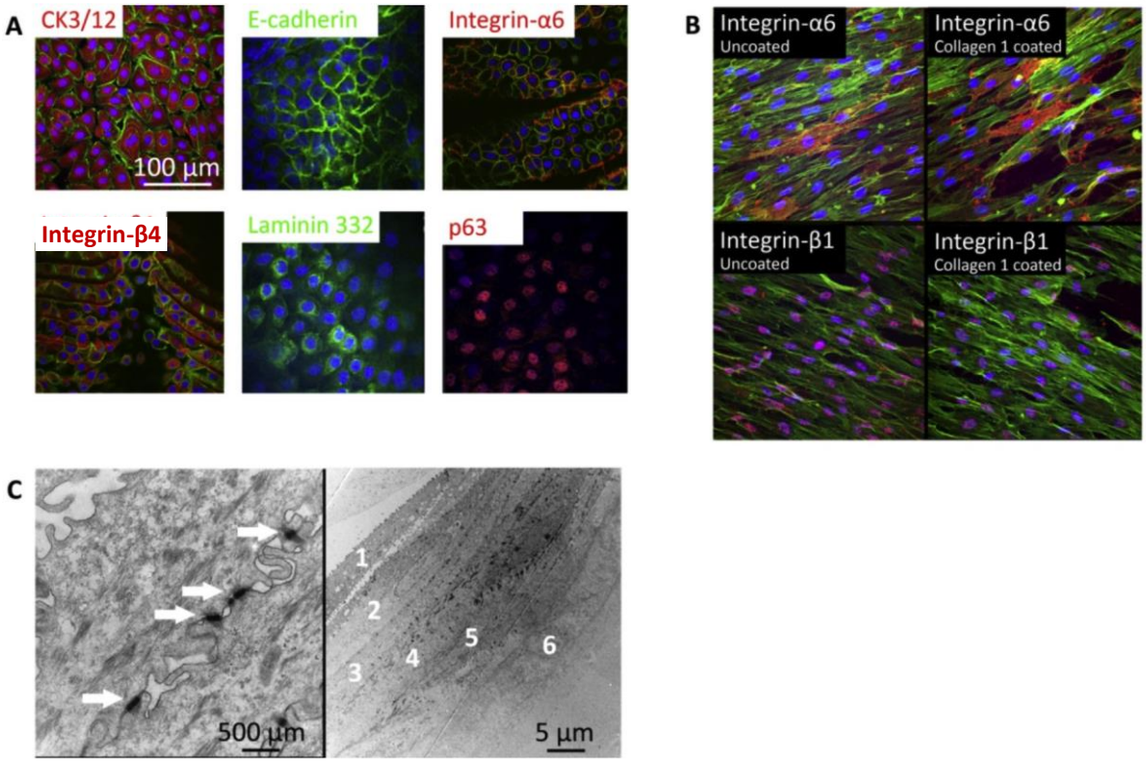

Figure 4. In vitro characteristics of primary human corneal epithelial cells grown on FSCM. A. Primary human corneal epithelial cells cultured on the FSCM stained positive for CK3/12 (red) (indicating differentiated epithelium), for the cellecell adhesion marker E-cadherin (green), for the cell-matrix/cellecell adhesion markers integrin-α6 (red), integrin-β4 (red), and basement membrane marker laminin 332 (green), and most cells stained positive for the stem cell marker p63 (red) (A; blue = cell nuclei; green = F-actin, unless otherwise stated). B. Primary human corneal stromal cells, the keratocytes, displayed the adhesion molecules integrin-α6 and integrin-β1, whether the FSCM was coated with collagen type I or not (B; blue = cell nuclei, green = F-actin, red = integrins). C. Desmosomes were present (arrows) between the epithelial cells cultured on the FSCM, and stratification occurred (C; numbers indicate cell layers).

Immunostaining for granulocytes, macrophages, T cells, and immunoglobulins showed more variation at 1 week postoperatively than after 6 and 11 weeks postoperatively. The composition of infiltrating immune cells one week after implantation was dominated by granulocytes, but also contained macrophages in all groups (Fig. 6B). The immune response at 1 week was graded as moderate and was not significantly different from the two control groups (Fig. 6C). The immune response diminished to mild and minimal over time until the end point at 11 weeks, for each of the four immunomarkers, and the response to the FSCM was again not different from that seen with the

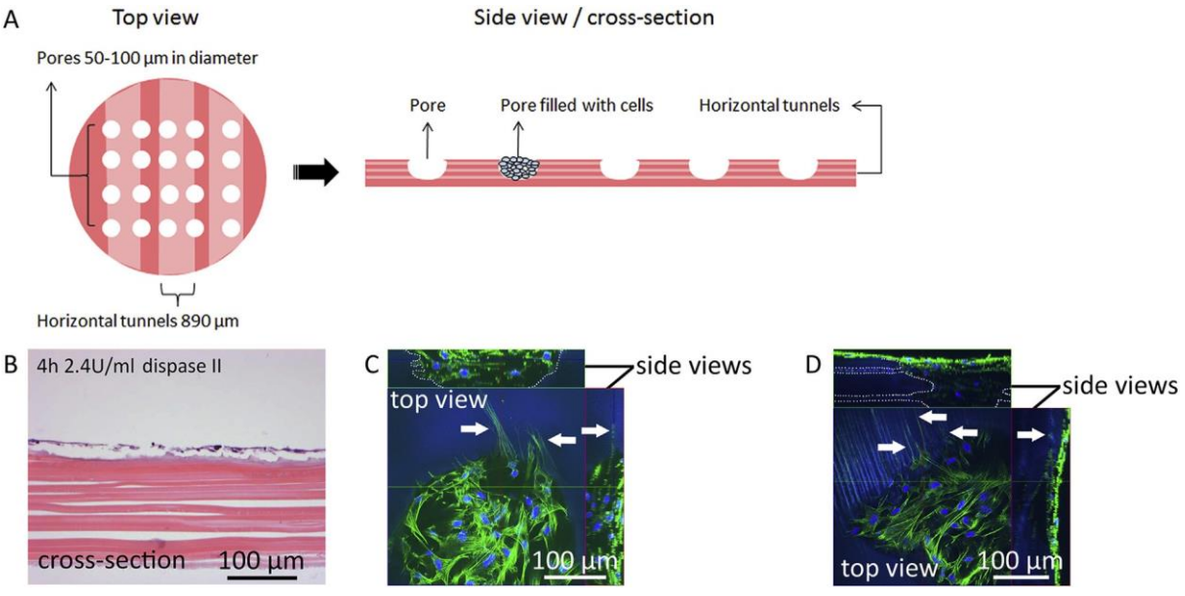

Figure 5. Modification of the FSCM by generating pores to increase cell ingrowth. The FSCM can be manipulated with the femto-second laser to create pores and horizontal tunnels in the FSCM (A), as well as with dispase II to create a small space between the naturally present horizontal collagen layers at the outer edge of the FSCM (B). Primary human corneal stromal cells are shown to interact with these alterations by filling up the small spaces (see arrows) thus created by dispase II in FSCMs with laser made pores (C), and in FSCMs in which horizontal tunnels were created subsequently (D). The white dotted lines indicate the borders of the pores (C) and of the pores and tunnels (D) created in the FSCM, and the side views represent digital cross sections.

control matrix (Ologen® Collagen Matrix), and sham surgery (Fig. 6C). Microscopic analyses revealed that the increased amount of macrophages present in the FSCM group at 1 week, and of granulocytes at 11 weeks, were due to false-positive counting of the folds on the sections for which the ImageJ software could not correct, thereby creating high error-bars. These samples were not excluded from the analysis. A subcutaneous immunological challenge with an FSCM, three weeks after the first exposure to the FSCM, elicited no increased immune response after 1 week (Fig. 6D).

Corneal compatibility

Finally, we evaluated the in vivo compatibility of the FSCM: curved FSCMs were implanted intrastromally in rabbit corneas to assess the corneal tissue response to the FSCMs. Rabbits that underwent sham surgery served as controls. The animals were observed for six weeks, and the corneas were subsequently analyzed histologically. Sutures were removed after 3.5 weeks as they gave rise to ocular irritation in both groups, which disappeared upon removal of the sutures. The cornea stayed clear throughout the follow up, and no epithelial defects or signs of melting were observed (Fig. 7A). Some corneal vascularization was present in the superior limbal area in both groups, but this was similar to the situation in the non-operated contralateral eyes (Fig. 7B). The

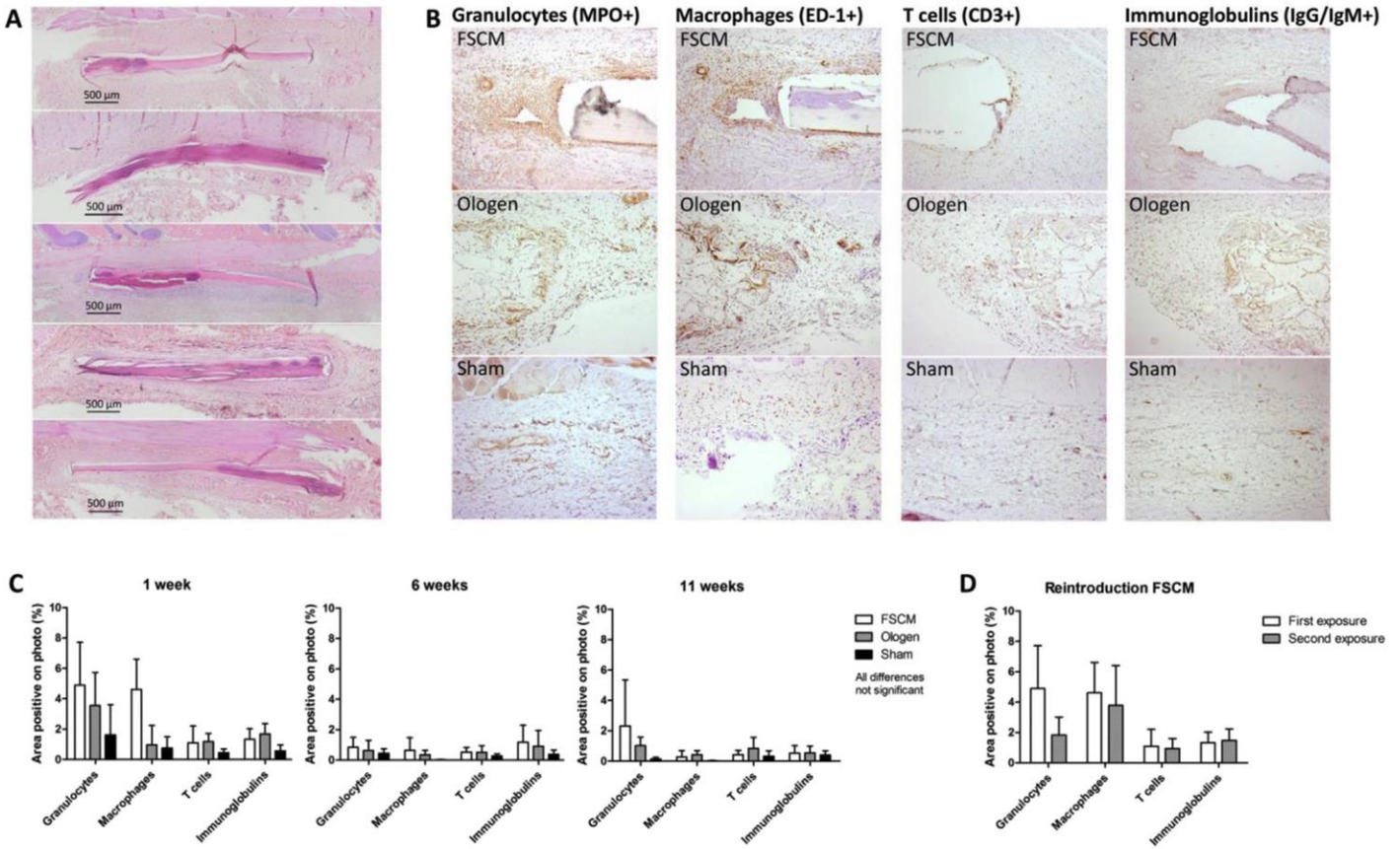

Figure 6. FSCM or control Ologen® Collagen Matrices were implanted subcutaneously and tissue were studied for different infiltrating cell types at 1, 6 or 11 weeks after implantation. A. Overview of the subcutaneous implanted FSCM and the surrounding tissue at 11 weeks on HE staining. B. Examples of the immunostaining pictures at one week follow up for granulocytes, macrophages, T-cells, and immunoglobulins for the FSCM, the Ologen® implant and following sham surgery. C. Staining areas were imaged by microscope (Zeiss Axioskop 40) and calculated with ImageJ. The graphs show the percentage of immunopositive staining areas for each marker, and demonstrate that all average values are below 5% and that they further diminish over time. D. After reimplantation, no sensitization has occurred as the immune response to the second exposure is similar to the first exposure. The tissue was tested 1 week after reimplantation.

corneal stroma showed a normal arrangement of its fibers around the FSCM. A few infiltrating cells were noticed around the edge of the FSCM (Fig. 7C). The infiltrate contained a mix of corneal stromal cells (keratocytes) and macrophages (Fig. 7D and E). No granulocytes were seen throughout the sections and in a few sections, a single T cell was observed. The activated cells were not seen to invade the FSCM, but appeared to deposit new extracellular matrix at the edges (Fig. 7D and E). The FSCM slightly pushed up the overlaying corneal stroma, resulting in a slight atrophy of the otherwise healthy epithelium covering the area positioned above some of the more superficially-implanted FSCMs, while showing normal epithelial morphology in the ones with deeper implants which caused less rising of the anterior stroma (Fig. 7C).

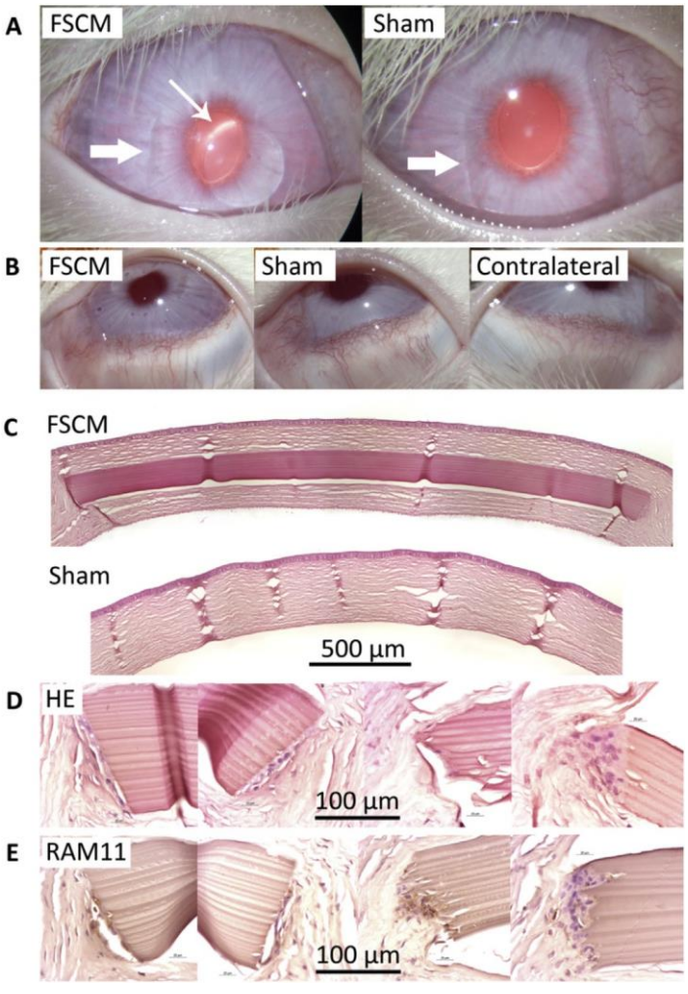

Figure 7. In vivo tolerance to FSCMs in rabbit corneas. A. Intrastromally implanted FSCMs (small arrow) in rabbits stayed clear during follow-up, and do not differ from the image of the rabbits that received sham surgery (big arrows indicate site of incision). B. Neovascularization was physiological and was equal in eyes with an FSCM to eyes after sham surgery or contralateral eyes. C. The overlaying epithelium appeared viable on the HE-staining, and a few infiltrating cells, depending on the location at the FSCM's edge (D), were present. E. Macrophages (RAM11 staining) were seen in the scarce infiltrate (dark brown is immunopositive).

The two rabbits implanted intrastromally with 6 mm diameter FSCM's for long-term follow up revealed no significant immune response as based on the in vivo follow up and the OCT images. The OCT revealed that there were no signs of extrusion of the implanted FSCM and that the thickness of the FSCM remained stable (Fig. 8).

Discussion

Our data show that the FSCM is suitable for use as basic material to develop an artificial cornea. It facilitates adequate epithelial regrowth and adhesion, is not immunogenic, and can be stably implanted in the cornea. 11.7 ± 0.9 MPa.

The glucose diffusion flux was maximal after 1 h (24.89 ± 6.78 x 10^{-6} cm^2/s). The glucose diffusion rate of the FSCM (24.9 ± 6.8 x 10^{-6} cm^2/s) is more than adequate, when compared to the human cornea (2.5-3.0 x 10^{-6} cm^2/s) [30]. The tensile strength (11.7 ± 0.9 MPa) is also nearly identical to that of the human cornea (3-9 MPa).[31]

It is of paramount that any material used as a basis to develop an artificial cornea has no unwanted effects that interfere with its purpose: to replace a damaged cornea for a given period of time. The material should be non-toxic to surrounding cells and not have a major influence on a cell's proliferative capacity. Our data illustrate clearly that the FSCM is neither cytotoxic nor has a major influence on epithelial proliferation. As the FSCM is being developed as a stromal replacement device, it should facilitate regrowth and adhesion of stable and healthy corneal epithelium. Our in vitro studies show that primary human corneal epithelial cells that grow on the FSCM are of the correct phenotype, i.e. expressing CK3/12, E-cadherin and desmosomes, the latter of which are both involved in cell-cell contact and communication between epithelial cells). Intact cell-cell contact and communication is very important in epithelial tissues to fulfill their barrier function.[32,33]

The presence of hemi-desmosomes and one of its components, the heterodimer integrin-α6 and integrin-β4,[34,35] shows that these cell-matrix adhesion molecules are produced by the epithelial cells, and indicates that cell-matrix adhesion is indeed present; this is essential for firm adhesion of newly-formed corneal epithelium to the FSCM. We also show the presence of laminin-322, as integrin-α6β4 preferentially binds to this protein, which is produced by epithelial cells and is part of the basal membrane, and strengthens the connection with the underlying collagen.[36] The finding that primary human epithelial cells are able to stratify when grown in specific medium on top of the FSCM greatly supports the use of this scaffold as a replacement therapy for corneal stroma.

Once the FSCM is implanted in the cornea, its border is adherent to the surrounding corneal stromal tissue. When the FSCM is sutured into place, the sutures should provide enough strength to hold it in situ. For long term use, the FSCM should become attached to the surrounding recipient's corneal tissue. In this adhesion process, the corneal stromal cells play a pivotal role, and our in vitro studies with primary corneal keratocytes show that they grow on the FSCM and express the cell-matrix adhesion molecules needed by keratocytes (Fig. 4B): integrins α6 and β1.[37]

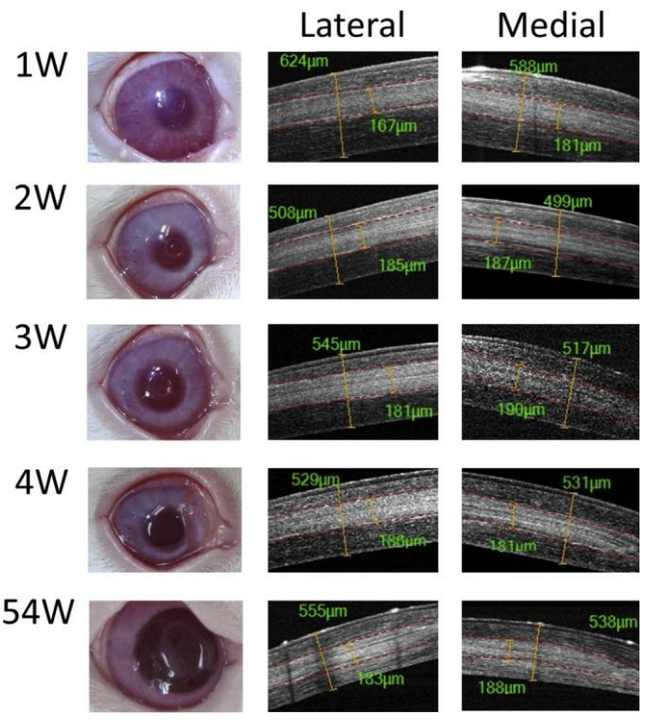

Figure 8. Long-term follow up of intrastromal FSCM's. The intrastromally placed FSCM remained stable in situ during a total of 54 weeks (54 W) without eliciting an immune response. The first column shows the rabbit eye in vivo, the second column shows a cross section made by OCT of the lateral area of the FSCM and the third column shows a cross section of the medial area of the FSCM. The thickness of the FSCM was stable and showed no signs of degradation. At 54 week some scar-tissue was visible.

In order to improve the physical interaction between the FSCM and the keratocytes, the FSCM can be modified using several techniques. We manipulated the FSCM with a femto second laser and dispase II, which led to additional attachment points for keratocytes. Additionally, cross-linking may be applied, a technique used to produce the curved FSCM, which was used for intrastromal implantation in rabbit corneas. That this FSCM is suited for post modification makes it ideal to adjust it as needed during its development towards an artificial cornea.

Implantation of the FSCM subcutaneously in rats allowed us to compare the immune response against the FSCM with the response against a collagen matrix that is already safely used in humans as well as with sham surgery: we demonstrated that the FSCM is not immunogenic, as there was no specific immune response, and there were no signs of sensitization upon reintroducing the FSCM subcutaneously in the same animal. This confirms our hypothesis as posed in our previous work,[26] namely that the inflammation following corneal placement in a pocket or as an anterior laminar graft was largely due to mechanical irritation. The loose rat skin still allowed some shifting of the FSCM, which is denser and harder than the spongy Ologen® Collagen Matrix, and in one case elicited fibrous encapsulation. Despite the possibility of movement of the matrix, and despite that the skin is

vascularized and not immunosuppressed, only a few immune cells and no specific immune responses were observed.

Our focus in the rabbit experiment, with intrastromal implantation of the FSCM, was to test whether the FSCM could have a negative effect on the cornea, i.e. melting of the overlying epithelium or eliciting an unwanted immune response during a longer follow up period (six instead of three weeks). For that reason we implanted a thinner and curved FSCM, thereby minimizing mechanical irritation. We used intrastromal implantation instead of an anterior lamellar keratoplasty, as we intended to determine the potential response elicited by the FSCM, and not by sutures or mechanical irritation. Using this model, we were able to place the sutures far away from the FSCM, which helped in distinguishing between a response elicited by and towards the sutures versus one elicited by the FSCM. We observed that all corneas stayed transparent, the overlaying epithelium remained healthy and stable during 6 weeks of follow-up, and on histology, a few infiltrating macrophages were observed; we can thus conclude that there were no unwanted adverse effects. Keratocytes were located near the skirt of the FSCM and appeared to interact with the exposed edges and we hypothesize that these keratocytes may be slowly replacing the FSCM's collagen type I by extra-cellular matrix deposition. The observed empty spaces between the layers at the FSCM's edge are considered to be due to the trephination. Long-term follow-up suggests that the FSCM will remain stable without extrusion, degradation or rejection for at least up to one year. Some scarring around the FSCM was visible however, which is something we should address.

When looking into the literature, there are several other solutions for corneal restoration. In contrast with the Boston Keratoprostheses, the FSCM does not need a donor cornea.[38] The OOKP is a complex but well researched solution as all other options for corneal restoration have failed.[39] The FSCM may offer an alternative.

A synthetic transparent scaffold (ACTO TexKPRO; polyvinylidene difluoride fibers and silicone) has successfully been implanted as preliminary treatment in six patients for 6-40 months after which a keratoplasty was performed. This ACTO TexKPRO is currently being developed as a solution for cases where the ocular surface has been destroyed.[40] A recent publication evaluated a hydrogel (polyethylene glycol/polyacrylic acid) designed as artificial cornea, also by intrastromal implantation in rabbits.[41] Fifty percent of the rabbits followed for 2 months (n = 6) kept clear corneas without inflammation. However, after up to 16 months follow up, there was a high rate of complications, such as epithelial defects, corneal thinning and even extrusion. Yet in a previous study, this hydrogel showed clear corneas up to around 6 months.[42] Our two rabbits had no extrusion or notable corneal thinning at 1 year follow up.

A clinically-used biosynthetic artificial cornea that is made from carbodiimide cross-linked recombinant human collagen, has been shown to be stable for up to 4 years of follow-up in 10 patients, although it resulted in thinner corneas (358 ± 101 mm).[16] This material is quite costly, and seems to be the closest to our material.

We showed previously that the FSCM has identical direct light transmission as the human cornea (although its light scatter values can be further optimized), and that it has a low immunogenicity. In our current study, we show that the FSCM is a scaffold that has no adverse effects to corneal cells in vitro, or to the cornea in vivo in the short term, and most likely also not in the long term. This clears the road for initiating larger longer follow-up experiments and to optimize its suturability. Further work will focus on the use of the FSCM as a corneal replacement strategy when no human material is available, and on the suitability of the FSCM as an emergency solution.

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CHAPTER 4

Matching for Human Leukocyte Antigens (HLA) in corneal transplantation - To do or not to do

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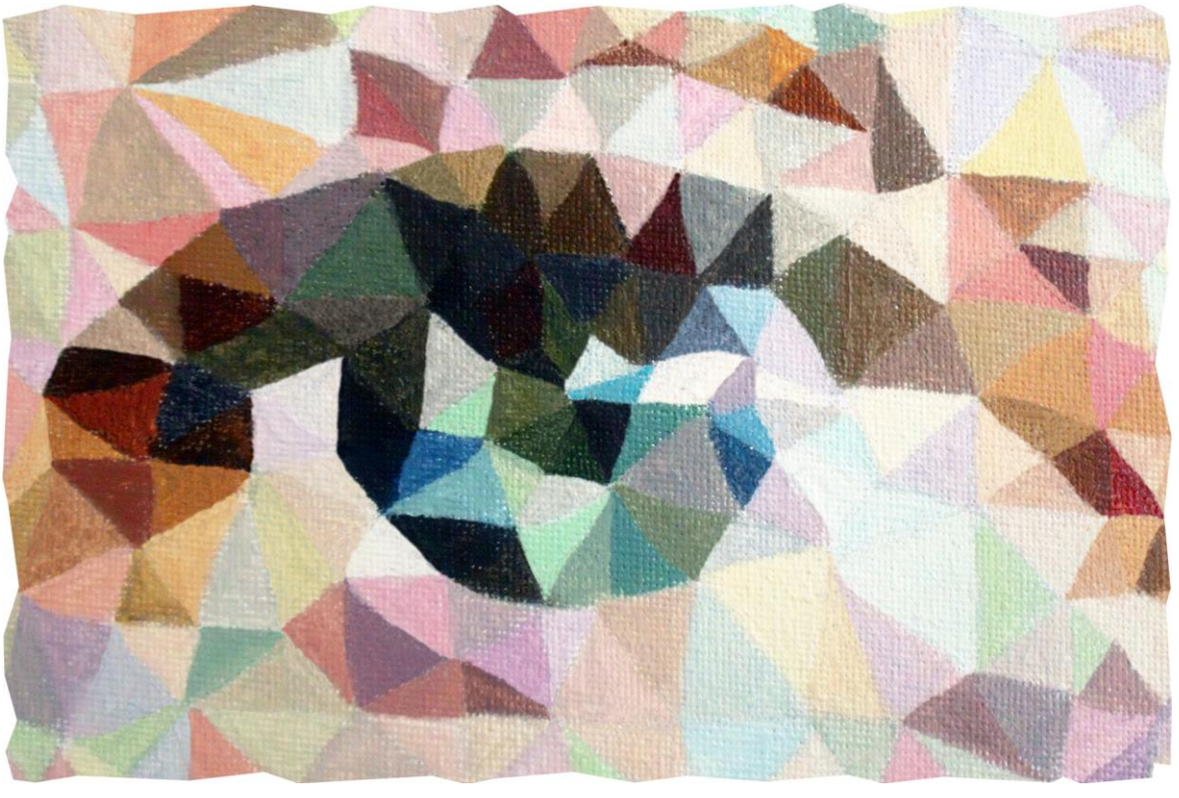

Abstract

As many patients with severe corneal disease are not even considered as candidates for a human graft due to their high risk of rejection, it is essential to find ways to reduce the chance of rejection. One of the options is proper matching of the cornea donor and recipient for the Human Leukocyte Antigens (HLA), a subject of much debate. Currently, patients receiving their first corneal allograft are hardly ever matched for HLA and even patients undergoing a regraft usually do not receive an HLA-matched graft. While anterior and posterior lamellar grafts are not immune to rejection, they are usually performed in low risk, non-vascularized cases. These are the cases in which the immune privilege due to the avascular status and active immune inhibition is still intact. Once broken due to infection, sensitization or trauma, rejection will occur. There is enough data to show that when proper DNA-based typing techniques are being used, even low risk perforating corneal transplantations benefit from matching for HLA Class I, and high risk cases from HLA Class I and probably Class II matching. Combining HLA class I and class II matching, or using the HLAMatchmaker could further improve the effect of HLA matching. However, new techniques could be applied to reduce the chance of rejection. Options are the local or systemic use of biologics, or gene therapy, aiming at preventing or suppressing immune responses. The goal of all these approaches should be to prevent a first rejection, as secondary grafts are usually at higher risk of complications including rejections than first grafts.

Contents

1. Introduction

Cornea transplantations are performed frequently, and results are often excellent. However, many cases are not eligible for a transplantation as the chance of rejection is considered too high. Rejection may even occur in low risk cases and in lamellar grafts, and one wonders whether matching for the HLA antigens may contribute to better graft survival. We review the evidence for this, especially focusing on studies in humans, and we looked which potential new approaches may be available to clinicians in the near future.

Since the first successful corneal transplantation in 1905,[Zirm, 1989] many improvements have been made that significantly increased graft survival. The development of microsurgery and the operating microscope allowed more precise corneal transplantations, and the recognition of the function of corticosteroids in the 1950s helped to reduce inflammation and fight potential rejection episodes. In 1982, a major textbook on immunology mentioned that "The cornea, the transparent membrane of the eye, is normally not perfused with blood and does not contain lymphatics, so it is not easily accessible to immune cells; when transplanted, it enjoys the status of a privileged graft. However, even in this favorable situation, graft rejection sometimes occurs, presumably because of damage to the grafting site inflicted by surgery".[Klein, 1982] It is clear that immunological theory and animal experiments led to the idea that corneas were hardly susceptible to rejection. Indeed, when looking nowadays at all cases, corneal allografting is one of the most successful forms of solid organ transplantation. Still, in spite of the great results in experimental corneal transplantation in rats, and the excellent results in certain groups of patients and types of corneal transplants, immune-mediated graft rejection remains the single most important cause of short- and long-term corneal graft failure. This is also the case when a cornea is implanted in normal- or low-risk patients.[Coster and Williams, 2005; Kuchle et al., 2002; Qu and Xie, 2010; Wilson and Kaufman, 1990] While overall survival rates for full thickness grafts are close to 90% one year after transplantation, they subsequently steadily decline.[Ing et al., 1998; Williams et al., 2006] At ten years, in random transplants, the overall graft survival rate is 60%-80%,[Coster and Williams, 2005; Ing et al., 1998; Inoue et al., 2000a; Thompson et al., 2003; Williams et al., 1997] with an immunological rejection rate after fifteen-years ranging from 21% to 29%.[Patel et al., 2004] Due to changes in surgical techniques, we now see fewer full thickness grafts, and anterior and posterior corneal replacements are gaining popularity, but rejection has been reported as still having a major impact, also in lamellar keratoplasties.[Allan et al., 2007; Mashor et al., 2010; Reinhart et al., 2011]

One assumed that matching of donor and recipient for HLA (human leukocyte) antigens was not necessary in corneal grafting due to the already protective ocular immune privilege; it is therefore not surprising that HLA matching studies were performed for kidney transplantation soon after the identification of the HLA antigens as a potential transplantation antigen system, but that HLA matching was only sporadically analysed for corneal transplants. When these studies were performed, the outcomes led to controversial results regarding the benefit of HLA matching to reduce corneal allograft rejection.[Baggesen et al., 1991; Beekhuis et al., 1991; Ehlers and Kissmeyer-Nielsen, 1979; Foulks et al., 1983;

Hargrave et al., 2004; Hoffmann and Pahlitzsch, 1989; Reinhard et al., 2003; Stark et al., 1978; The Collaborative Corneal Transplantation Studies Research Group, 1992; Vail et al., 1997; Vannas, 1975) A difference is especially observed between most European studies and the main study performed in the USA, the CCTS study. Subsequently, a long-term discussion on the benefit of HLA typing and matching and the strong opinions of some leaders in the field have discouraged research in this field, and one wonders whether that is correct. As every immune rejection starts with direct or indirect recognition of donor major or minor HLA-antigens as being foreign, there are still good reasons to unravel and not directly to disregard the role of HLA matching in corneal transplantation, and to properly evaluate the existing data.

It is essential to find ways to improve cornea graft survival in high risk cases, especially as a large group of patients with severe corneal disease is not even being considered for a human graft due the high risk of rejection, such as cases with severe burns or Stevens Johnson's disease. Limbal transplantation is not discussed in this review, but its application is severely limited due to the very high frequency of graft rejection.(Fernandes et al., 2004)

We will therefore address 1) the role of transplantation immunology in corneal transplantation, and specifically the role of HLA antigens in antigen presentation; 2) the influence of HLA matching in corneal graft survival, for both the major and minor histocompatibility antigens, and 3) discuss immunological approaches that may increase graft survival. We will focus on studies that may be clinically relevant in the near future, and will therefore limit the description of new basic research data in mice.

2. Corneal transplantation immunology

2.1. High and low risk cases

The cornea is the most-commonly transplanted tissue in humans, with over 65,000 transplants being performed worldwide each year.(Williams et al., 2009) Part of the success of corneal transplantations is based on the immune privilege of the cornea, and hence, corneal grafts have a survival benefit over organ allografts.(Niederkorn, 2001; Niederkorn and Stein-Streilein, 2010; Niederkorn, 2006; Skelsey et al., 2001; Streilein, 2003) Notwithstanding the irrefutable role that the immune privilege plays in the success of corneal grafting, it will not completely protect the unmatched graft from immune rejection, but only curtail the effect of immune responses. Graft survival is related to the status of the recipient bed, such as the amount of vascularization, and the underlying disease, and not so much to the recipient's age in the Australian data (Figs. 2-6). The latter finding is in contrast with other reports, such as Vail et al. (1997), who in a cohort of 2777 grafts studied in the UK Corneal Transplant Follow up Study observed less rejection with advancing age. While other causes of failure increased with age, graft survival did not differ. Vail's study showed that the risk of rejection was associated with the presence of glaucoma, inflammation, regrafting and large graft size. These associations point to the importance of the recipient bed: analyses of human corneas and animal studies have shown that the number of antigen-presenting cells in the receiving cornea play an important role: low- or normal-risk patients

have an avascular cornea, no inflammation and thus only a few antigen-presenting cells, and have long-term graft survival rates ranging from 62 to 96% after 10 years.[Williams et al., 1997] On the other hand, high risk patients with corneal vascularization in several quadrants of the cornea, active herpetic infection, and high densities of Langerhans' cells, have 10-year graft survival rates of 35%-41% [Thompson et al., 2003; Williams et al., 1997] Thompson et al. (2003) studied 3992 consecutive eyes that underwent a penetrating keratoplasty at a large tertiary care center, and reported that the most common causes of graft failure were endothelial failure and immunologic endothelial rejection. Keratoconus had the best 10-year survival, at 92%, while first regrafts had only 41% graft survival at 10 years. The presence of deep vessels at more than 3 clock hours led to 65% 10-year overall graft survival versus 82% in non-vascularized cases. As already mentioned, patients with highly-vascularized corneas are often not considered candidates for grafting, as their chances of developing rejection are too high. It is noteworthy that the transplants in the most successful cases, i.e. in keratoconus and primary endothelial dystrophy of Fuchs, are the conditions with the least amount of vascularization.[Jager et al., 1988] Retransplants are often placed in vascularized recipient beds, and every subsequent regraft has a lower chance of surviving.[Thompson et al., 2003; Williams et al., 1997]

If one also takes into account that upon failure of the primary graft, often a re-graft is performed, [Bersudsky et al., 2001; George and Larkin, 2004; Qu and Xie, 2010; Williams et al., 1997; Yahalom et al., 2005] it is obvious that reducing the risk of immunologic primary allograft rejection and understanding its mechanism is of vital importance to improve vision, to reduce graft loss and to prevent retransplantations.

2.2. Immune privilege

The immune privilege of corneal allografts is based on at least six physiological mechanisms, which either block the induction of the immune response, deviate the immune response towards a tolerogenic pathway or help to escape the immune attack:[Niederkorn and Larkin, 2010]

1) Absence of corneal lymph and blood vessels and blockade of lymph vessel formation, together with a relative lack of lymphatic drainage from the eye, ensures that antigens can only leave the eye via the aqueous draining system into the blood-stream.[Albuquerque et al., 2009] The aqueous outflow will carry antigens to the spleen, rather then the draining lymph nodes, which will then act as the primary lymphoid tissue.[Streilein, 1995]
2) Inducement of regulatory T cells by the allograft, which inhibits the induction and function of the alloimmune T cells.[Chauhan et al., 2009; Yamada et al., 2005]
3) Protection from complement-mediated cytolysis.[Bora et al., 1993; Hargrave et al., 2003; Hegde et al., 2002; Lass et al., 1990]
4) Induced apoptosis of neutrophils and T cells at the graftehost interface.[Stuart et al., 1997; Yamagami et al., 1997]
5) Diminished corneal T-cell proliferation[Hori et al., 2006; Jager et al., 1995; Shen et al., 2007] and,
6) Diminished NK cell activation.[Apte et al., 1998; Apte and Niederkorn, 1996]

Corneal immunosuppression is not a passive system based on the absence of antigen recognition, as was originally thought, but consists of active immunological interactions with many different players. The combined effect of these active mechanisms that inhibit immune response after introduction of antigen in the anterior chamber is known as anterior chamber-associated immune deviation.[Niederkorn, 2006; Stein-Streilein and Streilein, 2002; Streilein, 2003] Antigens placed into the anterior chamber of murine eyes are taken up by specific macrophages in the iris, which subsequently migrate to the spleen. The presence of TGF beta in the anterior chamber modifies the antigen-loaded macrophages in such a way that they develop immunosuppressive characteristics.[Wilbanks and Streilein, 1992] Leakage of donor corneal proteins into the anterior chamber of the eye may thus induce anterior chamber-associated immune deviation. Additionally, the compact architecture of the corneal stroma is believed to inhibit the infiltration of immune cells and the blood-aqueous barrier prevents immunologically-active cells and factors from entering the ocular tissue.[Streilein, 1995] Although functional antigen-presenting cells (APCs) are present in the peripheral and paracentral cornea, they are scarce and mostly immature in the healthy central cornea, resulting in weak local antigen presentation.[Mayer et al., 2007] As grafts are usually placed centrally, they encounter only a few APCs. This may of course be different when inflammation is present, bringing in massive numbers of APCs, as is the case e.g. in herpetic corneal infections.[Williams et al., 1989] Corneal tissue by itself is able to produce cytokines to inhibit T cell responses;[Jager et al.,1995] the nature of the factors that are involved has not yet been elucidated. In the clinical setting, a local immune response, elicited by the corneal transplantation, is further tempered by topical corticosteroids applied to the cornea post-operatively, supported (when needed) by oral immunosuppressive agents.[The Collaborative Corneal Transplantation Studies Research Group,1992] Mucosal tolerance can also be induced by conjunctiva-associated lymphoid tissue.[Dua et al., 1995]

2.3. Histocompatibility antigens

The first descriptions, of what is now known as the Human Leukocyte Antigen (HLA) system, the major histocompatibility complex (MHC) in humans, date from 1958.[Dausset, 1958; Payne and Rolfs, 1958; van Rood et al., 1959] As many ophthalmologists who perform corneal transplantations are not immunologists, we will include a description of the development of the HLA system, which explains its weird nomenclature.

In 1936, Peter A. Gorer, studying allogeneic tumor transplantation in mice, discovered the presence of an antigen (antigen II) responsible for rejection (Gorer, 1936); additionally, he found that the sera of mice, that had received an allogeneic tumor, contained antibodies against this tumor.[Gorer, 1937] In 1944-45, Peter Medawar confirmed that an immune response against the graft was responsible for rejection of allogeneic transplants.[Medawar, 1944, 1945] After the Second World War, Gorer continued his work, together with George D. Snell, and found that his antigen II was encoded by a gene on the H locus in mice.[Gorer et al., 1948] In 1958, Dausset (1958), van Rood et al. (1959), and Payne and Rolfs (1958) laid the foundation of what is now known as the HLA complex. They studied human sera obtained

from multi-transfused patients or multiparous women and noted that antibodies in these sera reacted with leukocytes from many other individuals, but not all, thus demonstrating the existence of a polymorphic system. The first named antigen was H2, for which Dausset gets the credits.[Dausset, 1958] Van Rood noticed that serum from a multiparous woman who developed a blood transfusion reaction contained antibodies against white blood cells instead of erythrocytes, and using sera from many women and a leukocyte agglutination assay, he identified a series of antigens. Van Rood reported the existence of a bi-allelic system of leukocyte antigens, which he called 4a and 4b (now known as HLA-Bw4 and -Bw6).[van Rood, 1962] Other researchers identified other leukocyte antigens, and when the International Histocompatibility Workshops were established, different researchers were able to compare their work. This led to the discovery that most of these leukocyte antigens were genetically closely linked, and inherited at one chromosomal region, which was originally called HL-A (Human Leukocyte, locus A),[Thorsby and Lie, 2005] with the letters forming also a combination of the H of Dausset's H2 locus, and Rose Payne's LA antigens.[Terasaki, 1990; van Rood, 1969] The chromosomal region that codes for the HLA antigens is located on the short arm of chromosome 6 and responsible for the polymorphic HLA complex. At the time it was discovered that there were different Class I loci,[Kissmeyer-Nielsen et al., 1968] many antigens had already been identified and given a number, and this explains the strange numbering of A's and B's (A1, A2, A3, Bw4, B5, Bw6, B7, B8, A9, etc). The terms Class I for HLA-A, -B and -C and Class II for HLA-DR, -DQ and -DP were introduced by Klein (1977) and Thorsby and Lie (2005).

The primary biological role of these HLA antigens is not to impede tissue matching (although the name histocompatibility antigens seems to suggest that this is the most important function of these antigens), but to present all kind of antigens to the immune system. Antigen presentation is important in the recognition of environmental pathogens such as viruses, bacteria and fungi, to which the direct innate immune system and subsequently the adaptive immune system must develop an adequate response. HLA antigens contribute to the induction of immune responses, as well as to the effector phase, by presenting target peptides to helper and effector T cells.

As already mentioned, HLA antigens are divided into two classes, HLA Class I and Class II. HLA Class I antigens are derived from the three classical loci HLA-A, -B and -C and the non-classical loci HLA- E, -F, -G, -H, -I and -J, and are expressed on platelets and almost all nucleated cells, except most cells of the central nervous system. The HLA Class I molecules present peptides, derived from proteins that are broken down inside the cell, to cytotoxic CD8- positive T cells. HLA Class II consists of three main genetic loci, HLA-DR, -DQ and -DP, and these molecules are expressed on some immunological cells such as B cells and activated T cells, and especially on antigen-presenting cells such as dendritic cells, macrophages, and monocytes, and on endothelial cells and thymic epithelial cells. The Class II molecules present peptides from exogenous antigens to the CD4-positive helper T cells. Cytokines can modify the level of expression of Class I and II molecules. The HLA genes are the most polymorphic genes in the human genome, providing a great diversity of HLA alleles, with each specific allele having the ability to present certain antigens better or worse than other alleles.

Transplanting tissue from one individual into another introduces a new set of donor HLA antigens into the recipient. The peptides that are derived from the foreign HLA molecules can either be presented by the patient's own HLA molecules, or by the donor's that are present in the graft (Fig. 1). Differences in the set of HLA alleles between donor and host may result in recognition of

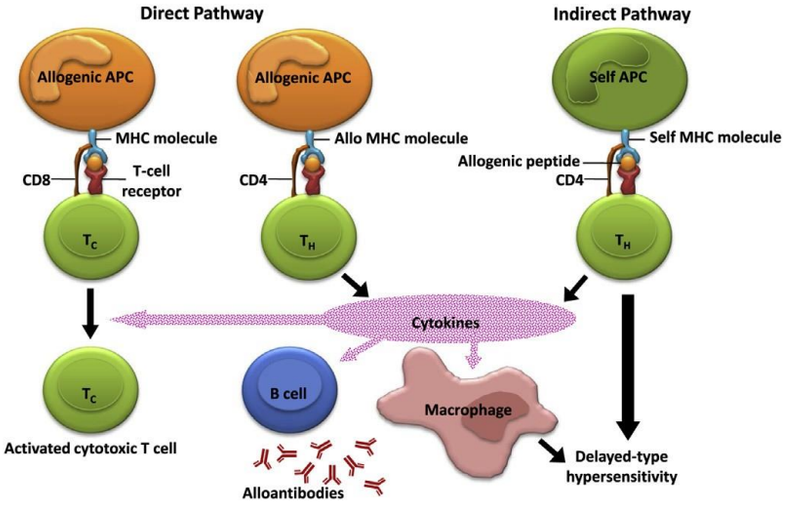

Figure 1. Pathways of recognition of HLA antigens (MHC) and mechanisms of graft rejection.

the donor tissue as foreign, and this may induce an immune response that ultimately ends in irreversible graft rejection. Reducing the differences in HLA antigens between donor and host by HLA matching should reduce the risk of rejection. HLA matching as a method to reduce graft rejection in living-related kidney transplantation came in use in the 1970s, when it was widely acknowledged that transplantation between HLA-identical living-related individuals was superior to other methods to improve graft survival.[Singal and Skinnider, 1970; Thorsby and Lie, 2005] Matching was first started using the HLA Class I antigens, but it took until 1978, after the identification of HLA-DR, before HLA-matching for cadaveric kidney transplantation showed beneficial results.[Albrechtsen et al., 1978a, 1978b; Opelz, 1985; Persijn et al., 1978; Ting and Morris, 1978]

In spite of all the mechanisms that are involved in the immune privilege of the eye, corneal graft rejection can occur and is caused by the immune response generated by recognition of host antigens. In spite of the early assumption that transplanted corneas would enjoy immunological privilege and could not be rejected, clinical data and early animal experiments demonstrated that especially grafts in vascularized recipients can be rejected.[Maumenee, 1951] Rejection can take place in all three distinctive corneal cell layers or in one layer only: the epithelium, stroma and endothelium. [Khodadoust and Silverstein, 1969] Knowledge about the distribution of the HLA antigens is therefore important to comprehend the rejection process. As graft rejection occurs most commonly in the endothelium,[Chong and Dana, 2008] one would expect this cell layer to have the highest expression of HLA antigens. This is not the case. HLA-A, -B and -C (Class I) are present at high levels on epithelial cells, especially near the limbus, at lower

levels on the stromal keratocytes and even less on the endothelium, although conflicting reports exist regarding the latter.[Baudouin et al., 1988; Pepose and Benevento, 1991; Treseler et al., 1984; Whitsett and Stulting, 1984; Williams et al., 1985)]

HLA-C is expressed at 10e35% of the levels of HLA-A or –B,[Bunce and Welsh, 1994; McCutcheon et al., 1995)] and is considered a weak transplantation antigen with no known role in matching for allografts. [Ferrara et al., 1978)] The Class II antigens HLA-DR, -DQ and -DP (Class II) are not expressed by the three corneal layers in the healthy eye; however, Class II positive epithelial dendritic cells are present, mainly in the peripheral corneal epithelium.[Baudouin et al., 1988; Pels and van der Gaag, 1984; Pepose and Benevento, 1991; Treseler et al., 1984; Whitsett and Stulting, 1984; Williams et al., 1985)] Studies on rejected human corneas show that inflammation leads to expression of HLA Class II in all cell layers.[Donnelly et al., 1985; Dreizen et al., 1988)]

Of the 'non-classical' HLA antigens (HLA-E, -F, -G, -H, -I and -J), only HLA-E (four alleles) and -G (three alleles) are known to be polymorphic and little is known about their expression and phys- iological role in the cornea. They have no known relevance to clinical corneal transplantation,[Taylor and Dyer, 1995)] although HLA-G is expressed in all three corneal layers.[Le et al., 2003)] Histocompatibility antigen expression is higher in corneas of younger persons compared with older persons.[Whitsett and Stulting, 1984)] Indeed corneal rejection risk is higher for grafts from young (0-5 years) than from older persons (40-70 years).[Palay et al., 1997)]

Aside from the major histocompatibility antigens, minor histocompatibility antigens (minor H antigens) exist. Minor H antigens are peptides presented by the HLA complex, and are derived from polymorphic proteins. A donor can have a different variant of the same protein as the host, which upon presentation of its peptides by HLA Class II molecules on donor or host APCs, will be recognized as foreign and can induce a strong immune reaction against the graft; this has been well documented in HLA-identical stem cell transplantations.[Dickinson et al., 2002; Goulmy et al., 1996; Goulmy, 2006)]

Known minor H antigens expressed on corneal tissue are HY, the male-specific minor H antigen, and HA-3.[de Bueger et al., 1992; Goulmy et al., 1995; Peeler et al., 1988; Ross et al., 1991; Sonoda and Streilein, 1992)] Animal studies have demonstrated that minor antigens can be an important target for immune responses in corneal transplantations. [Haskova et al., 2003; Sano et al., 1996, 1999)]

2.4. Immune rejection

Once a recipient's immune system recognizes the allograft as foreign, rejection will occur. In general, rejection can be defined as hyperacute, acute or chronic.[Bush, 1999)] Hyperacute rejection is the most rapid and aggressive form and takes place immediately after implantation. This type of rejection has been described in solid organ transplantation and is mediated by circulating antibodies, directed against ABO-blood group or HLA antigens or both; such antibodies may have developed as the result of a previous antigen exposure, such as a blood transfusion or pregnancy.[Wood and Goto, 2012]) With regard to corneal allografts, early studies found an influence of ABO incompatibility in high risk patients,[Inoue and Tsuru, 1999; Maguire et al., 1994)] but more recent studies did not confirm this. [Dunn et al., 2009; Soma et al., 2004; Stulting et al., 2012)] In contrast to the clear role which anti-HLA alloantibodies have in solid organ transplantation,

their role is not entirely clear in corneal transplantation. Although they can exacerbate a rejection, alloantibodies are not believed to be by themselves capable of acutely rejecting corneal grafts, most likely due to the lack of blood vessels in low risk grafts.[George and Larkin, 2004; Goslings et al., 1998; Stein-Streilein and Streilein, 2002] This minor influence of alloantibodies, together with the avascularity of the cornea, explains that hyperacute corneal rejection, if at all, seldom occurs. However, it should not be ignored that allosensitization as the result of previous antigen exposure still has an important influence on corneal transplant survival, even if it does not lead to acute rejection. The negative effects of immunization were found to be independent of the degree of vascularization.[Roy et al., 1992] Regrafting is undesirable as it leads to a higher chance of rejection and moreover, the fast occurrence of graft failure after a re-transplantation indicates the importance of absent prior allosensitization before grafting (Fig. 2).[Coster and Williams, 2005; Niederkorn and Larkin, 2010; Williams et al., 2012] The role of allo-antibodies in any type of corneal graft rejection is discussed below.

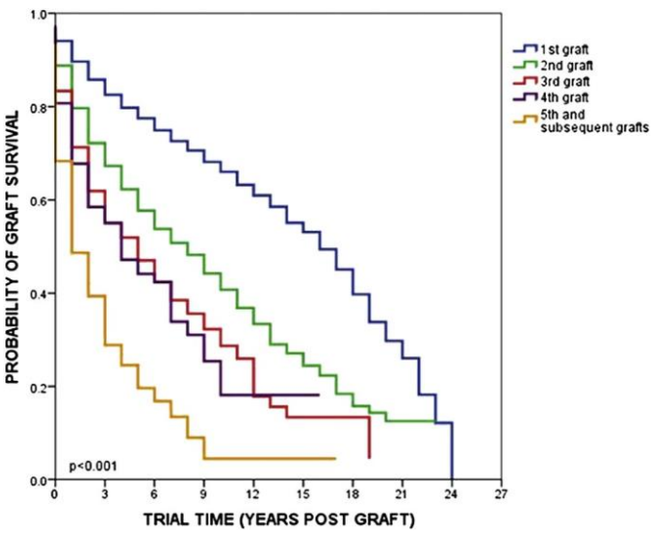

Figure 2. Kaplan-Meier survival plot for corneal graft survival according to the number of previously-rejected ipsilateral transplants. Regrafts have significant worse graft survival (log-rank test). Used with permission from Department of Ophthalmology, Adelaide: The Australian Corneal Graft Registry 2012 Report; KA Williams, MT Lowe, MC Keane, VJ Jones, RS Loh, DJ Coster (Eds); 2012, pp. 1-246.

Number at Risk

Identity	Initially	3 years	6 years	9 years	12 years	15 years	18 years	21 years	24 years
1st graft	12988	5441	2727	1359	754	360	119	32	1
2nd graft	2412	887	411	181	86	31	14	4	n/a
3rd graft	607	191	82	32	16	6	3	n/a	n/a
4th graft	161	51	25	11	3	2	n/a	n/a	n/a
5th & subsequent graft	123	30	7	2	1	n/a	n/a	n/a	n/a

Acute rejection manifests within days to months and is initiated by the recognition of new foreign antigens by the immune system: this type of rejection is antibody as well as cell-mediated.[Game and Lechler, 2002] With adequate and timely treatment, acute rejection can be resolved, although it can still predispose the host to chronic rejection.

Chronic rejection develops slowly and usually occurs after months or years and is believed to be mediated by antibody and cell-mediated immune responses with macrophage involvement.[Game and Lechler, 2002] Despite improvements in immunosuppressive therapies which can prevent and control acute rejection, chronic rejection is still a major problem, and has become the major cause of solid organ.[Bush, 1999; Racusen, 2003), and corneal allograft failure (Coster and Williams, 2005; Ing et al., 1998; Inoue et al., 2000a; Patel et al., 2004; Thompson et al., 2003; Williams et al., 1997, 2006)

With regard to the different corneal cell layers, epithelial rejection is, although probably common, generally quiet, asymptomatic, transient and usually does not affect graft survival.[George and Larkin, 2004; Khodadoust and Silverstein, 1969; Prendergast and Easty, 1991] Stromal rejection is relatively common, can progress to endothelial rejection if not treated, and can be acute or chronic,[George and Larkin, 2004; Khodadoust and Silverstein, 1969; Panda et al., 2007; Prendergast and Easty, 1991] as infiltrating stromal cells may persist for several years in the allograft.[Macdonald et al., 2010] Endothelial rejection can be acute or chronic, and is the most symptomatic and serious one of the three types of rejection.[Khodadoust and Silverstein, 1969] Endothelial cells persist for life as they are hardly proliferative. Therefore cell loss due to recurrent reversible rejection episodes may lead to graft failure due to endothelial decompensation.[Bourne, 2001; George and Larkin, 2004; Joyce et al., 1996; Prendergast and Easty, 1991)

Once the immune privilege of the anterior chamber and cornea has been compromised, a local immune response may lead to graft rejection. The loss of immune privilege may either be due to inflammation caused by the allograft itself, or to other causes of inflammation, corneal vascularization, or ocular surface diseases. A well-known trigger of rejection is a loose suture, which will attract mucous and bacteria and become a focus for leukocyte accumulation (macrophages, Langerhans' cells, T cells) inducing local upregulation of HLA antigens.[Jonas et al., 2002] Immune rejection involves both the innate and adaptive immune response. Major and minor H antigens can be recognized indirectly via presentation through the innate immune system and directly via the adaptive immune system as illustrated in Fig. 1.[Game and Lechler, 2002; Sayegh and Turka, 1998] The innate immune response (neutrophils, macrophages) on its own is not capable of rejecting the graft and the involvement of the adaptive immune response (T cells) is required.[Krensky et al., 1990; Niederkorn, 2007] The major bridge between the two is the interaction between APCs of the innate immune system and T cells of the adaptive system. APCs, of both the donor and host, are able to pick up material shed from the graft and activate the adaptive immune system by presenting these antigens to the CD4+ T helper cells. These CD4+ T helper cells play a central role in recruiting effector cells into the graft, as they are able to activate CD8+ cytotoxic T cells which subsequently attack the donor cells, and B cells which start producing antibodies against donor antigens. Furthermore, CD4+ T cells recruit

macrophages, granulocytes, and NK cells, and activate the complement system. All of these players are able to kill corneal cells, leading to rejection.

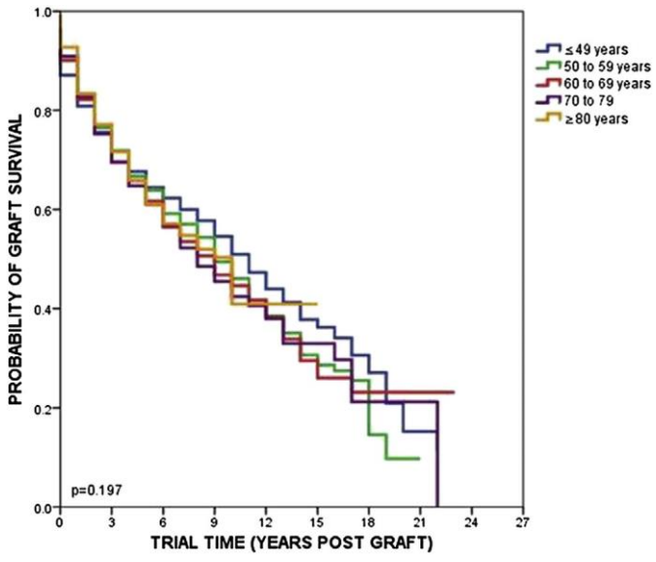

Figure 3. KaplaneMeier survival plot for corneal graft survival according to the recipient age (keratoconus excluded). Age at time of grafting had no significant effect on graft survival (log-rank test). Reprinted with permission from: Department of Ophthalmology, Adelaide: The Australian Corneal Graft Registry 2012 Report; KA Williams, MT Lowe, MC Keane, VJ Jones, RS Loh, DJ Coster (Eds); 2012, pp. 1-246. URL: http://hdl.handle.net/2328/25859.

Number at Risk

Identity	Initially	3 years	6 years	9 years	12 years	15 years	18 years	21 years
≤49 years	2060	868	454	235	142	73	26	5
50 - 59 years	1235	529	273	144	77	30	7	n/a
60 - 69 years	2102	907	471	224	109	34	5	1
70 - 79 years	3548	1316	541	176	62	14	2	1
≥80 years	2467	558	156	32	4	1	n/a	n/a

In the adaptive immune system, CD4+ T cells of the host respond directly to donor HLA Class II antigens, which are expressed on APCs, while CD8+ T cells of the host recognize donor HLA Class I antigens, present on the cell surface of donor corneal cells.[(Game and Lechler, 2002; Sayegh and Turka, 1998; Wood and Goto, 2012)]

Direct recognition plays a major role in acute rejection, while indirect recognition is additionally involved in acute and chronic rejection.[(Auchincloss et al., 1993; Lee et al., 2001; Sayegh and Turka, 1998)] Involvement of the adaptive immune response and especially of the T cells is required for corneal graft rejection,[(Krensky et al., 1990; Niederkorn, 2007)] but not necessarily simultaneously.[(Hegde and Niederkorn, 2000)] While studies in mice showed that minor H antigens may even have a more important role in rejection than major MHC antigens, very few studies have been performed to determine the presence of T cells directed against minor antigens in human corneal transplant rejections.

2.5. Cellular and humoral anti-corneal immune responses in humans

Once antigen presentation has occurred, immune responses against the graft may be induced.[(Maumenee, 1951)] It is possible that previous sensitization through an earlier graft or pregnancy has occurred, which has induced either an antibody or cellular immune response against the cornea, against minor antigens or against the HLA antigens. Many studies about the role of such immune responses have been performed in rabbits or mice, but only some studies regarding T cell or antibody responses are available regarding humans. In Leiden, crossmatches between recipient serum and cornea donor leukocytes have been performed since 1968, as antibodies were considered a risk factor for corneal graft rejection. Additionally, screening was performed in potentially-sensitized individuals to determine the presence of antibodies against a panel of leukocyte donors.[(van Rood et al., 1976)] This was initiated after early studies had shown that anti-leukocyte antibodies are present in cornea recipients. While one study showed the presence of directly lympho-cytotoxic T cells in the blood of corneal transplant recipients but hardly of any antibody,[(Grunnet et al., 1976)] a second study from the same group on one case correlated graft rejection with the presence of an antibody-dependent cell-mediated cytotoxicity response against peripheral blood lymphocytes.[(Ehlers et al., 1981)]

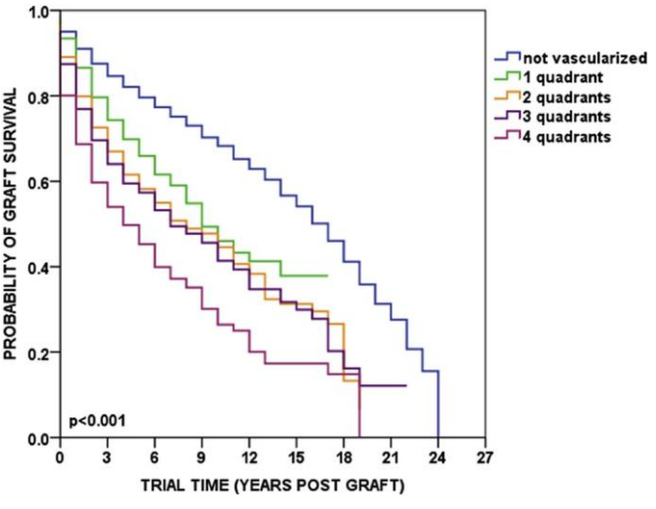

Figure 4. Kaplan-Meier survival plot for penetrating corneal graft survival according to the pre-graft vascularization status. Graft survival was shorter when more quadrants were vascularized (log rank test). Reprinted with permission from: Department of Ophthalmology, Adelaide: The Australian Corneal Graft Registry 2012 Report; KA Williams, MT Lowe, MC Keane, VJ Jones, RS Loh, DJ Coster (Eds); 2012, pp. 1-246. URL: http://hdl.handle.net/2328/25859.

Number at Risk

Identity	Initially	3 years	6 years	9 years	12 years	15 years	18 years	21 years
Not vascularized	11168	4871	2488	1266	703	336	123	34
1 quadrant	1158	417	182	60	22	7	n/a	n/a
2 quadrants	1721	614	267	136	71	25	4	n/a
3 quadrants	844	312	153	67	34	18	5	2
4 quadrants	1400	386	162	63	30	14	4	n/a

In the U.S.A., Stark collaborated with Terasaki, one of the first experts in identifying anti-HLA antibodies, to investigate the development of lymphocytotoxic antibodies in corneal transplant patients.[(Stark et al., 1973)] The presence of allo-antibodies was determined on a panel of leukocytes from 90 to 100 individuals in the microlymphocyte cytotoxicity test. Three cases had antibodies prior to transplantation, and all three developed a rejection.Five out of six patients who were negative prior to transplantation and developed a corneal graft rejection developed such antibodies, versus only one of eight patients who did not reject and had a successful transplant.[(Stark et al., 1973)] As this study clearly showed a correlation between a rejection and the development of antibodies and rejection, it

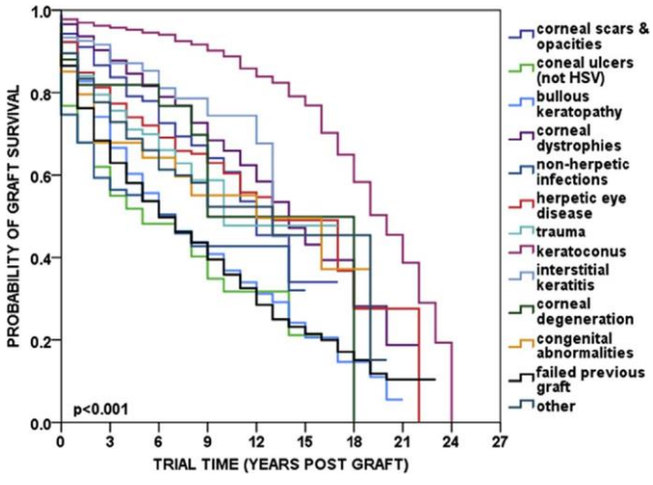

Figure 5. Kaplan-Meier survival plot for corneal graft survival according to main indications for penetrating grafts. The variation in survival across the main indications was significant (log rank test); keratoconus had the longest survival, and corneal ulcers the shortest. Reprinted with permission from: Department of Ophthalmology, Adelaide: The Australian Corneal Graft Registry 2012 Report; KA Williams, MT Lowe, MC Keane, VJ Jones, RS Loh, DJ Coster (Eds); 2012, pp. 1-246. URL: http://hdl.handle.net/2328/25859.

Number at Risk

Identity	Initially	3 years	6 years	9 years	12 years	15 years	18 years	21 years
Corneal scars & opacities	314	118	68	22	13	2	n/a	n/a
Corneal ulcers (not HSV)	349	71	32	15	6	1	n/a	n/a
Bullous keratopathy	3814	1236	475	168	62	20	4	1
Corneal dystrophies	1806	872	429	188	96	35	7	2
Non-herpetic infections	221	60	32	12	7	3	n/a	n/a
Herpetic eye disease	692	287	151	87	50	26	4	2
Trauma	266	103	54	21	9	3	n/a	n/a
Keratoconus	4930	2444	1367	781	471	251	98	27
Interstitial keratitis	151	81	41	19	11	n/a	n/a	n/a
Corneal degeneration	117	33	16	7	3	1	n/a	n/a
Congenital abnormalities	67	25	15	10	10	5	3	n/a
Failed previous graft	3277	1152	522	225	105	40	17	4
Other	287	112	56	30	17	11	3	n/a

led to a clear advice regarding testing of corneal transplant patients for the presence of cytotoxic antibodies: patients should be screened when there is a history of pregnancy, blood transfusion, or previous corneal transplant failure, upon which a cross-match test between the serum of the sensitized patient and cells from the potential donor should be performed, and mismatches of the same specificity as the cytotoxic antibodies of the recipient should be avoided. These criteria were not all based on scientific data from this study, but were similar to the screening advise given on the basis of data on kidney transplants.[Opelz et al., 1973]

Another study described that even in patients with 100% panel reactivity, acceptable mismatches could still be found and could be defined in 80% of these patients.[Vannas et al., 1976]

The Collaborative Corneal Transplantation Studies Research Group (1992) included a prospective study to compare donor-recipient pairs with (37 cases) and without (419 cases) a positive serological crossmatch (lymphocytotoxic anti-HLA antibodies): a higher frequency of graft failure due to rejection was seen in the cross-match positive group than in the crossmatch negative group.[Hahn et al., 1995] In another cohort, Des Marchais observed a negative influence of a positive crossmatch only in patients who had previously undergone a transplant or already rejected a cornea graft.[Des et al., 1998] In a study on 1681 consecutive keratoplasties, Volker-Dieben et al. reported that a panel reactivity of more than 10% led to a significantly-increased chance of rejection in moderately to severely vascularized recipients.[Volker-Dieben et al., 2000] When looking at an IgM crossmatch between patient serum and corneal donor rims, antibodies were found in 28% versus 25% of the recipients with preformed anti-HLA antibodies.[Sel et al., 2012] Although these studies provide some evidence that the presence of anti-HLA antibodies has an adverse effect on penetrating corneal transplants, too few studies have reported on their importance in different types of corneal diseases, or their importance for re-transplants after rejection. Recently, Sel et al. studied the impact of pre-existing donor-specific allo-antibodies against donor HLA Class I or II prospectively using a novel ELISA-based crossmatch procedure: in a short-term follow-up study, they found a 50% lower rejection rate in patients without allo-antibodies.[Sel et al., 2012] This is in accordance with the finding in solid organ transplantation that allo-antibody formation is related to acute and especially chronic allograft rejection.[Doxiadis, 2012; Terasaki, 2003]

Antibodies can be directed against major and minor HLA antigens or against corneal antigens. Nelken analyzed whether a corneal transplantation induced the development of antibodies that bound to corneal antigens, and indeed, in 15/33 cases such antibodies were observed.[Nelken and Nelken, 1965] Some studies analyzed responses against a specific soluble corneal protein, known as BCP 54 (Bovine cornea protein 54),[Jager et al., 1991] which has been identified to correspond to corneal aldehyde dehydrogenase (ADH).[Verjans et al., 1990] Antibodies against this antigen occur in healthy controls, but also in patients with Fuchs' heterochromic cyclitis or uveitis,[Kruit et al., 1985; van der Gaag et al., 1989] and such antibodies were found to be present in over 40% of cornea transplantation patients. Similarly, patients often displayed a T-cell mediated reactivity against this antigen.[Jager et al., 1994] No prognostic value could be attributed to the presence of a humoral or cellular anti-BCP 54 immune response, but

it was interesting that five of the six patients who had a rejection episode during the study period had anti-BCP antibodies prior to transplantation and changed from a negative to a positive cellular anti-BCP 54 response.(Jager et al., 1991) These studies suggest an increase in T cell reactivity against corneal antigens as the result of local inflammation, not necessarily as the cause of rejection. Furthermore, there may be a subgroup of patients who tend to develop any type of immune response.

Earlier, Stark et al. had used the leukocyte migration inhibition test to determine the presence of a cellular immune response in corneal graft recipients.(Stark, 1980) Only patients who had rejected a graft at the time of testing showed a positive response.

Roelen et al. determined the presence of cytotoxic T lymphocytes (CTLs) against mismatched donor Class I antigens in patients

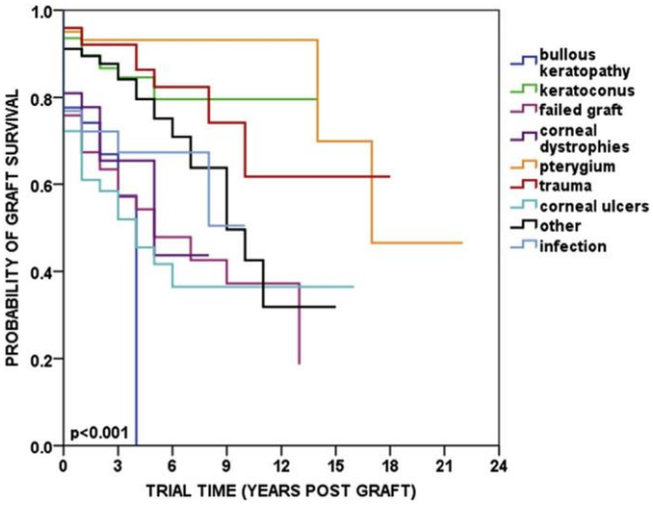

Figure 6. Kaplan-Meier survival plot for corneal graft survival according to the main indications for lamellar grafts. The variation in survival across the main indications was significant (log rank test); pterygium had the longest survival, and bullous keratopathy the shortest. Reprinted with permission from: Department of Ophthalmology, Adelaide: The Australian Corneal Graft Registry 2012 Report; KA Williams, MT Lowe, MC Keane, VJ Jones, RS Loh, DJ Coster (Eds); 2012, pp. 1-246. URL: http://hdl.handle.net/2328/25859.

Number at Risk

Identity	Initially	3 years	6 years	9 years	12 years	15 years	18 years	21 years
Bullous keratopathy	341	7	n/a	n/a	n/a	n/a	n/a	n/a
Keratoconus	266	41	12	4	2	n/a	n/a	n/a
Previous failed graft	252	30	12	8	3	n/a	n/a	n/a
Corneal dystrophies	126	5	1	n/a	n/a	n/a	n/a	n/a
Pterygium	163	48	17	11	7	3	2	1
Trauma	124	37	18	6	2	1	1	n/a
Corneal Ulcers	126	18	8	2	1	1	n/a	n/a
Infections	82	15	4	2	n/a	n/a	n/a	n/a
Other	270	74	18	9	2	1	n/a	n/a

with and without a corneal graft rejection. CTLs were divided into naïve and primed CTLs based on their sensitivity or resistance to anti-CD8 or cyclosporine A in vitro. Rejections were strongly associated with the presence of primed donor-specific CTLs.[Roelen et al., 1995]

All the different types of corneal transplantation and not only penetrating corneal transplants can lead to rejection as shown by data of the Australian Corneal Graft Registry for anterior (Figs. 7 and 8) and posterior keratoplasties (Fig. 9). Tan describes that in penetrating keratoplasty (PK), acute endothelial rejection occurs in 20% of the cases during 5-year follow up. While in anterior lamellar keratoplasty (ALK) there is no risk of endothelial rejection as the endothelium is not transplanted, stromal rejection occurs only in 1-2%. This lack of rejections is probably not directly related to the tissue characteristics, but to the disease, as ALK are mostly performed for keratoconus, a type of corneal disease that is not associated with corneal angiogenesis, and leads to infrequent rejection even in PKs. In endothelial keratoplasty (EK), endothelial rejection rates are probably similar as in PK. (Tan et al., 2012)

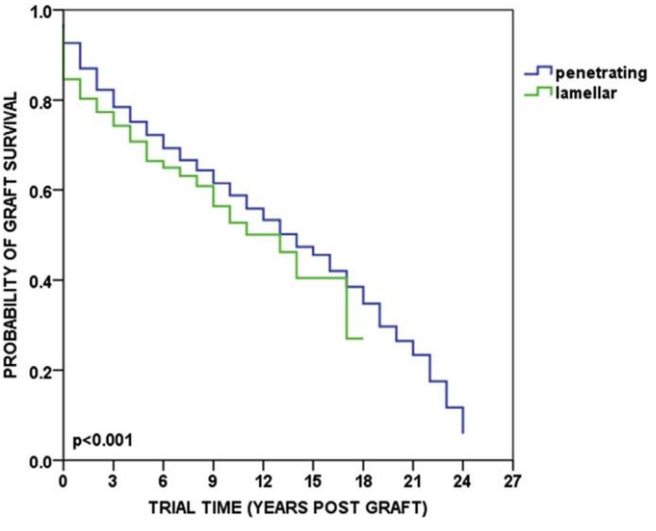

Figure 7. Kaplan-Meier survival plot for corneal graft survival according to type of keratoplasty (irregardless of indication). Penetrating grafts survived longer than the lamellar grafts (log rank test). Reprinted with permission from: Department of Ophthalmology, Adelaide: The Australian Corneal Graft Registry 2012 Report; KA Williams, MT Lowe, MC Keane, VJ Jones, RS Loh, DJ Coster (Eds); 2012, pp. 1-246. URL: http://hdl.handle.net/2328/25859.

Number at Risk

Identity	Initially	3 years	6 years	9 years	12 years	15 years	18 years	21 years
Penetrating	16736	6960	3428	1761	973	465	187	43
Lamellar	1751	274	90	41	16	5	2	n/a

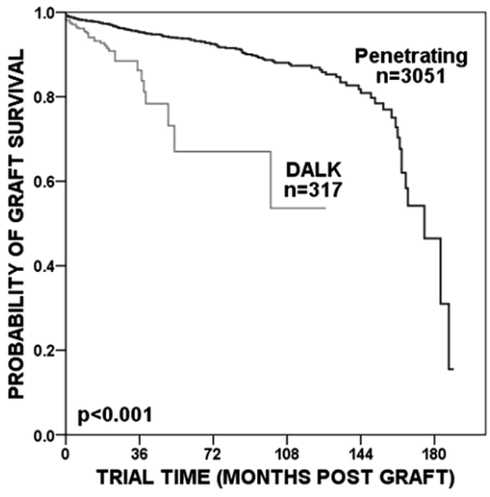

Figure 8. Kaplan-Meier survival plots of observed penetrating corneal grafts(penetrating) and deep anterior lamellar keratoplasty (DALK) procedures performed from 1996 through 2013 for keratoconus. The numbers on the plot represent the number of grafts at risk in each stratum. The differences between the curves are significant at P < 0.001 (log-rank test). Penetrating grafts for keratoconus fared significantly better than DALK procedures for the same indication over the same era. Reprinted from Ophthalmology, Vol. 121/5, DJ Coster, MT Lowe, MC Keane, KA Williams, A comparison of lamellar and penetrating keratoplasty outcomes: a registry study, pp. 979-987, Copyright 2014, with permission from Elsevier.

3. Matching major histocompatibility antigens

Data from the European Eye Bank Association in 1995 indicate that over time, HLA matching has been performed less and less, although some confounding factors are present. In 1991, matching for HLA Class I was performed in 24% and for Class II in 19% of the transplanted corneas, compared to 9.5% for both Class I and II in 1995.[(Maas-Reijs et al., 1997)] This trend continued and in 2008, HLA-Class II matching in corneal allografting was performed in only 3.3% of the transplanted corneas reported to the European Eye Bank Association.[(European Eye Bank Association, 2010)] However, a report by BISLIFE, based in the Netherlands, reported that 22% of the implanted corneas from centers they are collaborating with, were being HLA matched in 2009.[(BIS Foundation, 2009)] In the Australian Corneal Graft Registry Report of 2012, and the Cornea Donor Study published in 2012,[(Stulting et al., 2012)] HLA-matching rates and methods are not mentioned at all and are supposed to be performed rarely, even for corneal regrafts, while graft failure as an indication for corneal transplantation is increasing and immune rejection remains the main reason of graft failure. The Dutch Organ Transplantation Registry report of 2013 mentioned that of the 1286 transplanted corneas, only 46 were HLA-typed.[(Leiden van et al., 2014)] Currently, graft failure is the third or fourth indication for keratoplasty (both penetrating and lamellar), depending on the country, and ranges from 12 to 21%, while the percentage is gradually increasing overall, [(Keenan et al., 2012; Konijn-Janssen et al., 2011; Tan et al., 2014; Williams et al., 2012)] the number of grafts for Fuchs endothelial keratoplasty is increasing, and this may lead to more regrafts.[(Claesson and Armitage, 2013; Keenan et al., 2012)] This latter study compared first and regrafts for keratoconus, Fuchs endothelial

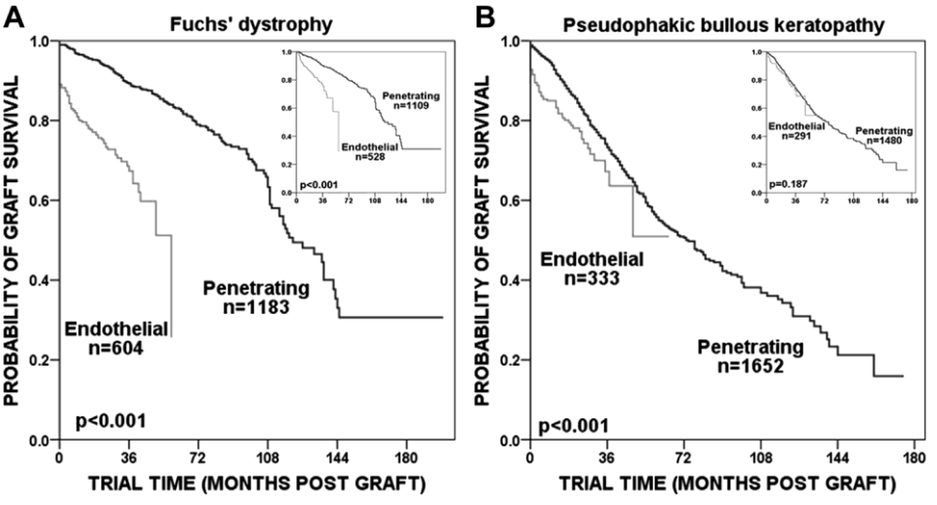

Figure 9. Kaplan-Meier survival plots of observed penetrating corneal grafts (penetrating) and endokeratoplasties (endothelial) performed from 1996 through 2013, stratified by indication for graft. The numbers on the plot represent the number of grafts at risk in each stratum. A, Grafts for Fuchs' dystrophy; B, Grafts for pseudophakic bullous keratopathy. The differences between the curves in (A) and (B) are significant at $P < 0.001$ (log-rank test). The insets show the data with all grafts that failed within the first post-operative month in each stratum that were removed from analysis. Penetrating grafts for Fuchs' dystrophy fared better than did endokeratoplasties for the same indication ($P < 0.001$), even when early graft failures were removed from analysis. Reprinted from Ophthalmology, Vol. 121/5, DJ Coster, MT Lowe, MC Keane, KA Williams, A comparison of lamellar and penetrating keratoplasty outcomes: a registry study, pp. 979-987, Copyright 2014, with permission from Elsevier.

dystrophy and bullous keratopathy in Sweden and demonstrated that not only the risk for failure was increased, but also the resulting visual outcome was worse when one needed a regraft for keratoconus and Fuchs endothelial dystrophy. One should thus look for ways to reduce the need for retransplantations.

One wonders whether more frequent HLA matching might help to prevent retransplantations. Matching is more expensive and cumbersome than the random distribution of corneal grafts. We looked at the papers that describe the effect of HLA matching on corneal transplant survival (Table 1), and while studies before 2000 have shown contradictory results for HLA matching for corneal allografts, the overall conclusion from more recent studies favours HLA matching for Class I for high risk cases (see below). However, the book has not been closed on the effect of HLA-DR typing and matching. (Baggesen et al., 1991; Beekhuis et al., 1991; Ehlers and Kissmeyer-Nielsen, 1979; Foulks et al., 1983; Hargrave et al., 2004; Hoffmann and Pahlitzsch, 1989; Reinhard et al., 2003; Stark et al., 1978; The Collaborative Corneal Transplantation Studies Research Group, 1992; Vail et al., 1997; Vannas, 1975) For example with regard to HLA-DR, the collaborative corneal transplantation studies (CCTS) found no beneficial effect of HLA-DR matching,(The Collaborative Corneal Transplantation Studies Research Group, 1992) while another study found that HLA-DR matching decreased graft survival.(Vail et al., 1994) A third study on the other hand found that matching for HLA-DR could inhibit or even obliterate the effect APCs have in initiating immune rejection.(Baggesen et al., 1996) The CCTS study was a major study performed in the USA,

and showed no positive effect of HLA-DR typing and matching in corneal allografts for HLA-A and -B either. However, when one looks closely at the HLA typing data of the CCTS, the outcome of the CCTS study was greatly affected by the lack of repeatability of the HLA typing: in a separate study, peripheral blood samples that had been used to determine the different HLA alleles in the CCTS were retyped, and it was noticed that there were especially problems with regard to HLA-DR.[Hopkins et al., 1992] As there was only 55% concordance between the original result and the outcome of retyping, this must clearly have prevented proper matching.[Hopkins et al., 1992; Volker-Dieben et al., 2000] Volker-Dieben et al. analyzed the effect of erroneous HLA-DR typing in a study where they determined the beneficial effect of HLA-DR typing on corneal allograft survival.[Volker-Dieben et al., 2000] Even with 5% erroneous typing results, the effect of matching for HLA-DR was lost. The conclusion of the CCTS study, i.e. that matching did not lead to better survival, was therefore based on incorrect typing data, and cases that were considered well matched, may well have been badly matched. Apart from the influence of erroneous matching, the rather aggressive immunosuppressive regimens used in this study possibly prevented any positive findings as well.[Armitage, 2004; The Collaborative Corneal Transplantation Studies Research Group, 1992; Volker- Dieben et al., 2000] One may wonder whether proper matching might reduce the amount of immunosuppression needed, or whether the use of aggressive immunosuppressive treatment overcomes the need for HLA matching in corneal transplantation. As corticosteroids may have many side effects, we will ask the question whether other studies support the use of HLA matching in corneal transplantation.

When we take into account the differences between low- and high- risk patient populations with a focus on recent findings, a positive effect of serological HLA-matching is certainly found for corneal allografting, and is most profound in the high risk patient group. The diversity in the reported results can be explained by the fact that in many studies, serological HLA typing was performed, which has been reported to be erroneous in 16-35% of the cases, and not the DNA-based HLA typing techniques which are much more accurate and came gradually into use around 2000.[Bozon et al., 1997; Yu et al., 1997; Zafar et al., 2003] The standardization of HLA typing techniques has been essential in improving results. While typing for HLA Class I antigens was well developed around 1978,[Albert et al., 1976; Terasaki et al., 1978] problems with HLA-DR typing were still encountered in 1992.[Volker-Dieben et al., 2000]

The application of DNA-methods to determine HLA gene poly-morphisms should be able to produce conclusive results on the benefit of HLA matching in corneal grafting. The influence of minor H antigens could be another factor explaining the contradictory results.[Nicholls et al., 1991]

3.1. Early studies on HLA matching

In 1986, a major study was published in the New England Journal of Medicine (Sanfilippo et al., 1986). This study reported on a prospective masked study at two hospitals, using ABO- compatible, crossmatch-negative recipients, matched for HLA-A and -B antigens. Only patients with vascularization of two or more quadrants or having a history of prior corneal-graft loss due to immune-mediated rejection were included. Post-operatively, topical corticosteroids were applied

Table 1. Overview of clinical studies of HLA-matching and corneal graft survival (details)

Author, year	Study setup	Patients (n)	Mean follow up	Antigens analyzed	Risk groups	Blood cross-matched before Tx	Typing method	Measured event
Allansmith et al., 1974	Random selected prospective Single center	43	12 months (range 0 – 26 months)	A	Overall	No (afterwards in 11 pt)	Broad Serological	Immunoreaction Non-immunological failure
Gibbs et al., 1974	Retrospective Single center	155	Unknown (observation period 24 months)	A	Based on degree of vascularization (absent, mild, severe)	No	Broad Serological	Rejection episode Opaque graft
Vannas, 1975	Prospective Single center	80	Unknown	A,B,C	Overall High (vascularized bed)	Yes	Both not mentioned (serological likely)	Rejection (not further defined)
Batchelor et al., 1976	Retrospective Single center	200	Unknown (range 7 - 44 months)	A, B	Overall Severe vascularization	No	Broad Serological	Irreversible immune rejection
Stark et al., 1978b	Prospective / *retrospective* Single center	84 / *19*	14 months (range 3-45 months)	A, B, C	High (<2 quadrants vascularized + regraft and/or previous foreign HLA exposure)	Yes / *No*	Broad Serological	Immunoreaction
Ehlers and Kissmeyer-Nielsen, 1979	Retrospective Single center	222	Unknown (range 0 - 138 months)	A, B	Overall	Unknown	Broad, typing method unknown (serological)	Rejection episode and/or opaque graft
Foulks and Sanfilippo, 1982	Prospective Singe center	46	16 months (range unknown)	A, B	High (>1 quadrant vascularized and/or regraft)	Yes	Broad Serological	Graft failure
Ozdemir, 1986	Prospective Single center	40	24 months (range unknown)	A, B	High (>1 quadrant vascularized and/or regraft)	Yes	Broad Serological	Graft failure
Sanfilippo et al., 1986	Blind prospective, Two center	97	Unknown (range 1 – 77 months)	A, B	High (>1 quadrant vascularized and/or regraft)	Yes	Broad Serological	Immunoreaction and irreversible reaction
Keyserlingk et al., 1987	Retrospective One center	57	22 months (range unknown)	A, B, DR	Normal High (significant vascularization, prior rejection episode, corneal perforating ulcer)	Unknown	Broad Serological	Immunoreaction and graft failure
Volker-Dieben et al., 1987	Prospective Two center	1218 /*123*	Unknown (range 12-121 months, end point Kaplan Meier 60 months)	A, B /*DR*	Overall High (> 1 quadrant vascularized)	Unknown	(Broad) Serological	Clear graft
Hoffmann and Pahlitzsch, 1989	Prospective, Single center	137	Unknown	B, DR	Normal High (based on preoperative diagnosis, postoperative AC irritation, graft diameter > 7.5mm, less than 2 compatible B or DR)	Unknown	Not mentioned	Endothelial immunoreaction
Boisjoly et al., 1990	Masked prospective Single center	435	36 months follow up for all	A, B	Overall	Yes	Broad Serological	Endothelial immunoreaction Graft failure

Baggesen et al., 1991 Study updated (extended) in 1996	Prospective Multi center	74	Unknown (range 1 - 40 months)	DR	High (vascularization >0 quadrant and/or regraft)	Unknown	Broad and DNA, Serology and RFLP (DNA)	Graft failure
Beekhuis et al., 1991	Prospective (A, B), *Retrospective (DR),* Single center	107	Unknown (at least 36 months)	A, B /*DR*	High (> 1 quadrant vascularized and/or regraft)	Unknown	Unknown	Immunoreaction as cause of graft failure
The Collaborative Corneal Transplantation Studies Research Group, 1992	Double blind prospective Multi center	419	Unknown (at least 18 months, and for 69% at least 36 months)	A,B, DR	High (> 1 quadrant vascularized)	Yes, but unknown whether for all	Broad Serological	Immunoreaction and graft failure (due to rejection and due to all causes separately)
Hoffmann et al., 1994	Retrospective Single center	248	Unknown (at least 18 months)	A,B, DR	Normal High (regraft)	Unknown	Serological	Immunoreaction
Vail et al, 1994	Prospective Multi center	542	Unknown (range 7 -66 months)	A, B, DR	Low (Normal) High (based on recipients age, regraft, diagnosis, vascularization, surgeon experience)	Unknown	Not mentioned	Immunoreaction and graft failure
Baggesen et al., 1996	Masked, randomized retrospective Multi center	74	Unknown (at least 36 months)	DR	High (vascularization >0 quadrant and/or regraft)	Unknown	High resolution RFLP (DNA)	Graft failure
Vail et al., 1997	Retrospective Multi center	602	Unknown	A, B, DR	Overall	Unknown	Not mentioned	Immunoreaction
Munkhbat et al, 1997	Retrospective Single center	81	Unknown (1 year follow up)	DRB1, DQB1, DPB1	Low (normal) High (> 2 quadrant vascularized and/or regraft)	Unknown	Broad RFLP (DNA)	Immunoreaction
Munkhbat et al, 1999	Retrospective Single center	79	Unknown (1 year follow up)	A, B	Low (normal) High (> 2 quadrant vascularized and/or regraft)	Unknown	Broad RFLP (DNA)	Immunoreaction
Volker-Dieben et al, 2000	Prospective / *retrospective* Single center	1681/*558*	58 months (range unknown)	A, B / *DR*	Low (Normal) High (> 1 quadrant vascularized)	Yes	Broad, Serological	Immunoreaction
Khaireddin et al, 2003 (Update on study Hoffmann et al., 1994)	Retrospective Single center	459	31 months low risk (range 2-159 months) 45 months high risk (range 2-182 months)	A, B, DR	Low (normal) High (>1 quadrant vascularized and/or previous ulcer, burn, or regraft)	Unknown	Broad and splits Serological	Immunoreaction
Bartels et al., 2003	Prospective Single center	303	50 months (median; range unknown)	A, B	High (>1 quadrant vascularized and/or regraft)	Yes	Broad (matching) and split (analysis) Serological (complement-depended cytotoxicity)	Immunological graft failure (graft not clear in 2 months with treatment) Overall graft failure

Reinhard et al, 2004a	Prospective Single center	418	40 months (not 60 months as reported; range unknown)	A, B, DR	Low (normal)	Unknown	Broad Serological (A, B) Immunogenetical (DR)	Immunoreaction Rejection-free clear graft
Böhringer et al., 2004	Prospective Single center	545	24 months (range unknown; standard deviation 18 months)	A, B, DR	Low (normal) (Avascular and first transplant)	Unknown	Broad (matching) and splits with HLA Matchmaker (analysis) Serological (A, B) Molecular (DR)	Immunoreaction
Minor antigens								
Inoue etal., 2000b	Retrospective Single center	396	49 months normal risk (range 6-122 months) 45 months high risk (range 6-123 months)	H-Y	Low (normal) High (>1 quadrant vascularized and/or regraft)	Unknown	Gender based	Immunoreaction
Reinhard et al, 2004a	Prospective Single center	418	40 months (not 60 months as reported)	H-Y	Low (normal)	Unknown	Gender based	Immunoreaction
Böhringer et al., 2006 (Reinhards group)	Retrospective Unknown amount of centers	291	31 months for H-Y (range unknown; standard deviation 22 months) 37 months for HA-3 (range unknown; standard deviation 22 months)	H-Y, HA-3	Low (normal) High (oversized grafts, glaucoma, herpetic scars, and/or regraft)	Unknown	Gender based (H-Y) and by PCR (HA-3)	Immunoreaction

two to three times per day. The well-matched recipients developed a rejection episode in 21% of cases, versus 49% in the poorly-matched group.

According to a report published in 1995,[Beekhuis, 1995] cornea transplant centers in the Eurotransplant area (The Netherlands, Germany, Belgium and Austria) used HLA-matched grafts in 19% of cases. However, between centers, this percentage varied between 0 and 64%, and there was no consistency in the weighing of the importance of the HLA-A and -B versus the HLA-DR antigens. The views on what constituted a high-risk cornea for which HLA matching was advantageous were fairly consistent: a previous immunological rejection in the same eye, deep stromal vascularization in two or more quadrants, and chemical burns. There was no consensus on whether a regraft in the same eye after failure without a rejection, or a previous immunological rejection in the fellow eye constituted an indication for HLA matching. In Germany, HLA-DR compatibility was considered more important than matching for the Class I antigens, while in the Netherlands, only matching for HLA-A and -B was applied.[Beekhuis, 1995]

3.2. HLA matching using HLA-A, -B and -DR

Matching donor and recipient can be performed for HLA Class I only, where two A's and two B's can be matched. When also looking at HLA-DR antigens, the total number of potentially-matchable antigens goes up to six. In 2000, Volker-Dieben et al. reported on all corneal transplantations performed in a single center between 1976 and 1996, that had been matched on the basis of HLA-A and -B antigens, and retrospectively typed for HLA-DR (558 cases).[Volker- Dieben et al., 2000] A beneficial effect of HLA-A and -B matching was found in both non-vascularized and vascularized cases, and a very significant influence was noticed for 0 mismatches for HLA-DR versus 1 or 2 mismatches for HLA-DR in moderately and severely vascularized recipients. No effect of DR matching on non- vascularized cases was noticed. Surprisingly, the paper does not provide information which technique was used for HLA-DR typing, serology or PCR.

Studies performed since 2000, which mostly use modern methods of HLA typing, show a clear beneficial effect of HLA matching in (high-risk) patients.[Bartels et al., 2003; Hargrave et al., 2004; Khaireddin et al., 2003; Osawa and Streilein, 2005; Reinhard et al., 2003; Volker-Dieben et al., 2000] Khaireddin, using serological HLA typing, observed that a donor-recipient match of two or more of the six alleles in HLA-A, -B or -DR reduced the rejection rate by at least 10% in low-risk (at 10 years after PKP) cases, and by 40% in high-risk patients, at three years after PKP. An analysis at the split antigen level (highly detailed HLA typing) showed no improved results. In the low-risk group, matching for either the HLA-B or -DR locus was found to reduce the number of rejections, while in the high-risk group matching for any of the loci reduced rejections. In 2001, Bartels et al. performed retrospective DNA-based HLA typing of donors and of recipients with or without a rejection episode.[Bartels et al., 2001] An increased rejection-free survival time of the patients (low- and high risk combined) was observed in graft recipients with one or two HLA-A matches, while no difference was observed for HLA-B and -DR. However, when one specifically looked at the high-risk group and studied the split level typing (identifying HLA subtypes),

less rejection was observed in graft recipients with one or two HLA-DR matches. HLA-B matching had no significant effect on graft rejection, which may be due to the low number of HLA-B matches observed in this retrospective study (as the B locus is the most polymorphic one).[Bartels et al., 2001] In 2003, Bartels repeated the study for only HLA-A and -B, looking at the split-level HLA typing in a larger group of high-risk patients,[Bartels et al., 2003] taking graft failure instead of graft rejection as end-point. Still, a better survival was found when there was no or only one split-mismatch for HLA-A/HLA-B.

In 2004, Reinhard et al. reported on a study in 418 first keratoplasties with an avascular recipient cornea, in which matching took place if a cornea with 0e2 mismatches of the six HLA-A, -B and -DR antigens could be found within 6 months.[Reinhard et al., 2004a] If a match had not been found during that time period, a graft with 3e6 mismatches was allocated. HLA typing was performed serologically for the Class I antigens, and by PCR for Class II; corticosteroids had been given topically as well as systemically. Even in these low-risk cases, a clear influence of matching was observed: at 4 years postoperatively, 92% of the matched group and 72% of the badly-matched group were rejection free. Most of these rejections were reversible; nevertheless, a positive effect on corneal clarity was present. Interestingly, Reinhard et al. did not find a clear influence of the number of (mis)matches on chronic endothelial cell loss in a study of 223 normal risk transplantations. However, as only first PKPs in an avascular host cornea were included in their study, the occurrence of immunological responses was limited (although no numbers were reported) and strangely, cases with an identifiable immune response during follow up were excluded from the study.[Reinhard et al., 2004b] Bohringer et al. reported in a prospective single center study of 545 normal risk corneal transplants, matched for HLA-A, and -B at the split level with a HLAMatchmaker algorithm (triplet string matching), that having 13 or less mismatches led to 85% rejection-free graft survival at 3 years, which significantly differed from the 76% rejection free survival when having more than 13 mismatches.[Bohringer et al., 2004] This was despite having significantly more HLA-DR mismatches in the first group. Conventional matching for HLA-A and -B resulted in 92% rejection-free survival when only having 0e1 mismatches compared to 76% rejection-free survival when having 3-4 mismatches. Although this last finding was not significant, these studies clearly state the benefit of HLA-A and –B matching. An overview of the studies found in literature on HLA matching is shown in Tables 1 and 2.

The unclear effect of HLA-DR may be due to different outcomes in relation to the degree of Class I matching: in kidney trans- plantation, HLA-DR matches lead to increased graft survival, but the otherwise significant effect of HLA-A and -B matching disappears when HLA-DR is incompatible.[Doxiadis et al., 2007; Johnson et al., 2010]

3.3. Approaches to allo-antibodies

Although cell-mediated immunity is considered the dominant cause of corneal allograft rejection,[Boisgerault et al., 2001; Hegde et al., 2005; Krensky et al., 1990; Niederkorn, 2001, 2007] complement-activating allo-antibodies may also contribute to corneal allograft rejection, especially once sensitization has

occurred. In contrast to patients awaiting a renal transplant, patients on the corneal transplant waiting list are not routinely screened for the presence of HLA-specific antibodies. Serum of only high-risk or retransplant patients is screened for such antibodies, using either the complement-dependent cytotoxicity assay and Elisa (LAT, One Lambda) or the luminex screening assay (Lifecodes® SSO; Immucor, Nijlen, Belgium). Patients with a negative screening result (Panel Reactive Antibodies (PRA) 5%) are identified as non-immunized. For corneal transplant patients, pretransplant crossmatches are not being performed. In patients that are immunized (PRA >5%), the complement-dependent cytotoxicity test is used to characterize the specificities of the HLA antibodies. In the few immunized patients, one thoroughly looks for acceptable mismatches, first by selecting HLA antigens which gave negative results in the complement- dependent cytotoxicity screening and by applying the HLA Match-maker program as described by Duquesnoy.[(Duuquesnoy, 2002)]

3.4. Current strategy for HLA screening and typing

In the Netherlands, only high-risk recipients (mostly retrans- plants) are typed for the HLA antigens: for the LUMC, on average only five patients per year. In contrast, a considerable number of cornea donors (around 250 a year) happen to be typed for HLA in the LUMC for other organ donation purposes. The HLA class I an- tigens are currently typed using oligonucleotide probes, that pro- vide a low resolution result with names that follow the HLA nomenclature that was developed in 2010 by the WHO Committee for Factors of the HLA System. HLA-DRB1 and DQB1 are similarly genotyped using the sequence-specific oligonucleotide probe (PCR/ SSOP) technique as previously described.[(Verduyn et al., 1993)]

HLA typing of the donors is used for allocation of these corneas to high risk patients and is done on basis of HLA-A, -B and -DR antigen matching, taking broad specificities into account first. Furthermore, in immunized patients, unacceptable mismatches are reckoned with and donors are selected after exclusion of these HLA mismatches. The allocation rules are ordered on HLA-A, -B, -DR broad level, country of the recipient, urgency, and HLA-A, B antigen split level. Currently, 60 Dutch patients are on the Dutch waiting list for an HLA-typed cornea, as well as 150 patients from abroad (Germany, Belgium, Austria, Italy, and Scandinavia). Around 600 Dutch patients are waiting for a non-HLA typed cornea. Annually, around 1250 Dutch inhabitants receive a non HLA-typed cornea transplant and around 50e60 a HLA-typed cornea transplant (data provided by the Dutch Transplantation Society).

3.5. HLA Matchmaker

HLA Matchmaker is a matching algorithm which was originally introduced to explain why many mismatched transplants do well. HLA Matchmaker determines histocompatibility at the epitope rather than antigen level in terms of the humoral alloimmune response. An epitope has two characteristics namely antigenicity, i.e. the reactivity with antibody, and immunogenicity, i.e. the ability of inducing an antibody response. Immunogenicity depends on the structural difference

between an immunizing protein and the antibody responder's homologous proteins. Certain structural differences lead to immunodominant epitopes whereas others are associated with low immunogenicity. The elucidation of three-dimensional molecular structures and amino acid sequence differences between HLA antigens made it possible to view each HLA antigen as a string of short linear sequences (triplets) involving polymorphic amino acid residues in antibody-accessible positions. These triplets are considered key elements of epitopes that can induce the formation of specific antibodies.

The triplet-matching concept has clinical relevance because HLA-A, B mismatched kidney transplants that are compatible at the triplet level have practically the same graft survival rates as the zero HLA-A, B antigen mismatches defined by conventional criteria. Triplet matching has been shown to benefit platelet transfusions of refractory thrombocytopenic patients. HLA Matchmaker is also useful in the determination of acceptable mismatches for highly-sensitized patients that are considered for kidney transplantation.

Recent studies have led to an updated version of HLA Matchmaker: stereo-chemical modelling of crystallized complexes of antibodies with different protein antigens revealed that antigenic proteins have functional epitopes consisting of amino acid residues that are about 3 Å apart from each other and at least one of them is non-self. The term now used to describe patches of polymorphic residues within a radius of 3.0-3.5 Å is “eplet”. By using this updated version of HLA Matchmaker (using eplets) a more complete repertoire of structurally-defined HLA epitopes can be made, which also provides a more detailed assessment of HLA compatibility.

Using HLA Matchmaker[(Duquesnoy, 2002)] to identify the number of mismatched HLA Class I eplets (polymorphic amino acid configurations), Bohringer et al. were able to show an additional value of the HLAMatchmaker on top of HLA Class I matching in preventing immunorejection after keratoplasties.[(Bohringer et al., 2010)]

4. Matching minor histocompatibility antigens

Even in fully HLA-matched cases, corneal survival, after excluding the non-immunological causes for graft failure, is well below 100%.[(Baggesen et al., 1991; Beekhuis et al., 1991; Ehlers and Kissmeyer-Nielsen, 1979; Foulks et al., 1983; Hargrave et al., 2004; Hoffmann and Pahlitzsch, 1989; Reinhard et al., 2003; Stark et al., 1978; The Collaborative Corneal Transplantation Studies Research Group, 1992; Vail et al., 1997; Vannas, 1975)] This indicates that aside from major histocompatibility antigens, minor H antigens could play a role. Studies in murine models in the 1990s observed that minor H antigens were of higher relevance for alloimmunity leading to corneal graft rejection than HLA antigens.[(Nicholls et al., 1991; Sano et al., 1996; Sonoda and Streilein, 1992; Yamada and Streilein, 1998)] In humans, one study found that mismatching for the minor HY antigen, which is only expressed on male cells, seemed not to influence graft survival.[(Inoue et al., 2000b)] However, an influence of HY in this study might well been obscured as they did not correct for HLA-antigen mismatches. A more recent clinical study on corneal graft survival, with a mean follow up of two years and taking HLA matching into account, showed

that mismatches for the minor H antigen HY indeed led to more graft rejection. Mismatches in the minor antigen HA-3 also had a negative effect on graft survival, although not significantly.[Bohringer et al., 2006]

Since both the HY and HA-3 minor H antigens are expressed on a variety of tissues, including corneal tissue, it justifies a potential role for both in corneal transplantation,[de Bueger et al., 1992; Dierselhuis and Goulmy, 2009; Goulmy et al., 1995] and especially for HY, as larger solid organ transplant studies found increased graft loss for HY mismatches;[Gratwohl et al., 2008] gender mismatches are known to be an independent risk factor in HLA-matched Stem Cell Transplantations.[Gratwohl et al., 2001; Stern et al., 2006] More research in this area is warranted, as we do not yet know which other polymorphic tissue-specific antigens are expressed in the cornea, and more specifically, from which proteins these peptides have been derived.

5. Other approaches to prevent rejection

5.1. Potential of biologics to prolong human corneal graft survival

Most of the recent studies indicate that matching donor and recipient for HLA-A and -B antigens may improve graft survival in high risk cases, and probably in low risk cases as well. However, when this is not possible, one may try to reduce the chance of rejection using a variety of techniques. Knowing the degree of mismatching may help to decide on the post-transplant protocol. One of the new classes of drugs that may help to improve corneal graft survival is that of the biologics, which have especially been applied experimentally, but are increasingly being used clinically. Biologics, which in this context are primarily recombinant antibodies or fusion proteins, are an important and widely-used class of drugs for the treatment of many ocular conditions including in- flammatory eye disease,[Durrani et al., 2011; Jap and Chee, 2008] diabetic macular edema,[Ho et al., 2012] and neovascular age-related macular degeneration.[Holz et al., 2014]

The choice of route of administration of biologics is a complex issue that is of direct relevance to prevention and treatment of clinical corneal graft rejection. Unmodified protein drugs cannot be delivered orally, because they will be rapidly degraded in the gastrointestinal tract. In humans, the common routes of administration of biologics for amelioration of eye disease include intravenous injection (used for example to deliver infliximab in some cases of Behçet's disease), and intravitreal injection (used to deliver eg. bevacizumab, ranibizumab or aflibercept to moderate aberrant angiogenesis and reduce macular edema in the posterior segment).

Table 2. Overview of clinical studies of HLA-matching and corneal graft survival (outcome)

Author, year	Antigens analyzed	Outcome (n = group size)
Allansmith et al., 1974	A	**No effect overall** (immunoreaction); no p-value mentioned 2 matches 14% immunoreaction (n = 7); 1 match 9% immunoreaction (n = 22); 0 matches 0% immunoreaction (n = 14) NB: effect of confounding factors not mentioned.
Gibbs et al., 1974	A	**No effect overall** (rejection episode); no p-value mentioned 2 matches 41% rejection (n = 29); 1 match 52% rejection (n =62); 0 matches 41% rejection (n = 64) **Beneficial severe vascularization** (opaque graft with or without rejection); p < 0.05 2 matches 47% opaque graft (n = 19); 0 matches 76% opaque graft (n = 29) NB: effect of other confounding factors not mentioned.
Vannas, 1975	A,B,C	**Beneficial overall** (rejection); no p-value mentioned 5-6 matches 4% rejection (n = 27); 3-4 matches 21% rejection (n = 19); Untyped 26% rejection (n=34) **Beneficial high risk** (rejection); no p-value mentioned 5-6 matches 8% rejection (n = ?); Untyped 39% rejection (n = ?) NB: effect of confounding factors not mentioned.
Batchelor et al., 1976	A, B	**Beneficial severe vascularization** (irreversible immune rejection) Two year follow-up (n = 73): 2 matches 27% rejection (n = 12); 1 match 72% rejection (n = 30); p < 0.05 2 matches 27% rejection (n = 12); 1 or 0 matches 70% rejection (n = 60); p < 0.01 Confounding factors (unknown whether equally distributed per group): 120 first grafts and 80 regrafts; graft sizes differ 5-10 mm;
Stark et al., 1978b	A, B, C	**No effect high risk** (immunoreaction); no p-value mentioned 4 matches 0% immunoreaction (n = 1); 3 matches 40% immunoreaction (n = 5); 2 matches 9% immunoreaction (n = 11); 1 match 26% immunoreaction (n = 35); 0 matches 27% immunoreaction (n = 51) Confounding factors (unknown whether equally distributed per group): 20 first grafts and 64 regrafts.
Ehlers and Kissmeyer-Nielsen, 1979	A, B	**Beneficial overall** (rejection episode and/or opaque graft); p < 0.005 2-4 matches 12% rejection (n = 49); 0-1 matches 35% rejection (n = 173) Confounding factors (having impact on outcome and not corrected for): Transplant indication, p-value unknown.
Foulks and Sanfilippo, 1982	A, B	**Beneficial high risk** (graft failure); trend, no p-value mentioned 3-4 matches 0% graft failure (n = 7); 0-2 matches 21% graft failure (n = 39) NB: effect of confounding factors not mentioned.
Ozdemir, 1986	A, B	**Beneficial high risk** (graft failure): p < 0.05 Five year follow-up: 2-3 matches 15% graft failure (n = 20); 0-1 matches 45% graft failure (n = 20) NB: effect of, or possible confounding factors not mentioned.
Sanfilippo et al., 1986	A, B	**Beneficial high risk** (immunoreaction); p < 0.01 (Chi-square Test), p < 0.01 (Multivariate Cox Regression) 2-3 matches 21% immunoreaction (n = 38); 0-1 matches 49% immunoreaction (n = 59) **Beneficial high risk** (irreversible immunoreaction); p < 0.02 (Multivariate Cox Regression) For the irreversible immunoreactions, group size and percentage are unknown. NB: p-values of multivariate analysis adjusting for age and graft size.
Keyserlingk et al., 1987	A, B, DR	**Beneficial high risk** (graft survival); p = 0.001 2-4 matches 0% graft failure (n = 7); 0-1 matches 46% graft failure (n = 13) **No data for normal risk** (n = 20) NB: effect of, or possible confounding factors not mentioned.
Volker-Dieben et al., 1987	A, B /*DR*	**A, B** **Beneficial overall** (graft clarity); p < 0.001 2-4 matches (A,B) ± 40% opaque graft (n = 497); Untyped ± 50% opaque graft (n = 721) **Beneficial high risk** (graft clarity); p < 0.001 2-4 matches (A,B) ± 45% opaque graft (n = 397); Untyped ± 60% opaque graft (n = 343) ***DR*** **No effect overall** (graft clarity); ns 2 matches (DR) ± 12% opaque graft (n = 45);1 match (DR) ± 25% opaque graft (n = 64); 0 matches (DR) ± 28% opaque graft (n = 14) **No effect high risk** (graft clarity); ns 2 matches (DR) ± 18% opaque graft (n = 34); 1 match (DR) ± 34% opaque graft (n = 44); 0 matches (DR) ± 30% opaque graft (n = 11)

		Confounding factors (having influence on graft clarity, but not corrected for): degree of vascularization, p < 0.001; number of regrafts, p < 0.001; diagnosis, p < 0.001; patient age, p < 0.001; graft diameter, p < 0.001 NB: unknown whether the 123 DR-typed patients were in the matched or untyped group for A,B.
Hoffmann and Pahlitzsch, 1989	B, DR	**Beneficial overall** (immunoreaction); p < 0.05 2-4 matches 11% immunoreaction (n = 46) vs. 0-1 matches 29% immunoreaction (n = 91) **No effect normal risk** (immunoreaction); p < 0.10 2-4 matches 0% immunoreaction (n = 15); 0-1 matches 13% immunoreaction (n = 68) **Beneficial high risk** (immunoreaction); p < 0.01 2-4 matches 16% immunoreaction (n = 31); 0-1 matches 74% immunoreaction (n = 23) NB: effect of confounding factors not mentioned.
Boisjoly et al., 1990	A, B	**Beneficial overall** (immunoreaction); p < 0.001 (multivariate analysis correcting for vascularization, graft diameter, HLA-DR, regrafting, and age) 2-4 matches 19% immunoreaction (n = 174) (0 or 1 mismatch at both loci); 0-2 matches 40% immunoreaction (n = 261) (2 mismatches at either loci) **Beneficial overall** (graft failure); p = 0.04 (unclear whether corrected for confounders) 2-4 matches 18% graft failure (n = 174) (0 or 1 mismatch at both loci); 0-2 matches 29% graft failure (n = 261) (2 mismatches at either loci) Analysis for DR and high risk group had too less power.
Baggesen et al., 1991 Study updated (extended) in 1996	DR	**Beneficial high risk** (graft failure); p = 0.003 18 month follow-up 1-2 matches 7% graft failure (n = 51); Untyped 50% graft failure (n = 23) Confounding factors (having influence on outcome, but not corrected for): regrafts, p < 0.01; NB: RFLP detected mismatches were serology did not
Beekhuis et al., 1991	A, B /*DR*	**Unknown, no statistical comparisons made** NB: More grafts failed from non-immunological reasons than from immunoreactions: 13 out 33 due to immunoreaction
The Collaborative Corneal Transplantation Studies Research Group, 1992	A,B, DR	36 months follow-up: **A, B** **No effect high risk** (graft failure all causes); p = 0.59 3-4 matches 33% graft failure (n = 137); 0-2 matches 37% graft failure (n = 282) **No effect high risk** (immunoreaction); p = 0.83 3-4 matches 64% immunoreaction (n = 137); 0-2 matches 66% immunoreaction (n = 282) **No effect high risk** (failure due to immunoreaction); p = 0.66 3-4 matches 21% failure due to immunoreaction (n = 137); 0-2 matches 26% failure due to immunoreaction (n = 282) **DR** **No effect high risk** (graft failure all causes); p = 0.99 2 matches ± 43% graft failure (n = 199); 0-1 match ± 41% graft failure (n = 220) **No effect high risk** (immunoreaction); p = 0.53 2 matches ± 69% immunoreaction (n = 199); 0-1 match ± 70% immunoreaction (n = 220) **No effect high risk** (failure due to immunoreaction); p = 0.87 2 matches 25% failure due to immunoreaction (n = 199); 0-1 match 24% failure due to immunoreaction (n = 220) **Beneficial high risk** (failure due to immunoreaction); p = 0.02 When using only donor-recipient pairs in which four distinct DR antigens were identified. NB: p-values of multivariate analysis adjusting for ABO, vascularization, and regrafts
Hoffmann et al., 1994	A,B, DR	**A** **No effect normal risk** (immunoreaction); ns 1-2 matches 11% immunoreaction (n = 109); 0 matches 13% immunoreaction (n = 56) **No effect high risk** (immunoreaction); ns 1-2 matches 56% immunoreaction (n = 59); 0 matches 54% immunoreaction (n = 24) **B** **Beneficial normal risk** (immunoreaction); p < 0.01 1-2 matches 4% immunoreaction (n = 80); 0 matches 19% immunoreaction (n = 85) **Beneficial high risk** (immunoreaction); p < 0.01 1-2 matches 47% immunoreaction (n = 64); 0 matches 84% immunoreaction (n = 19) **DR** **Beneficial normal risk** (immunoreaction); p < 0.05 1-2 matches 10% immunoreaction (n = 122); 0 matches 16% immunoreaction (n = 43) **Beneficial high risk** (immunoreaction); p < 0.05 1-2 matches 49% immunoreaction (n = 68);0 matches 87% immunoreaction (n = 15) NB: postoperative regimen changed during the years
Vail et al, 1994	A, B, DR	**A, B** **Beneficial overall** (graft failure?); trend, not significant

		DR **Bad effect overall** (graft failure?); p = 0.02 NB: unclear which groups were compared, no data on amount of immunoreaction/graft failure per HLA loci, DR groups not corrected for HLA-A,B differences and visa versa.
Baggesen et al., 1996	DR	**Beneficial high risk** (graft failure); p = 0.03 2 matches 23% graft failure (n = 38); 0-1 matches 42% graft failure (n = 36) Confounding factors (having influence on outcome, but not corrected for): Regrafts, p < 0.05;
Vail et al., 1997	A, B, DR	At 12 months follow-up: **A, B** **Beneficial overall** (immunoreaction); trend, not significant due to too small groups **DR** **Bad effect overall** (immunoreaction); no p-values mentioned NB: unclear which groups were compared, no data on amount of immunoreaction/graft failure per HLA loci, DR groups not corrected for HLA-A,B differences and visa versa.
Munkhbat et al, 1997	DRB1, DQB1, DPB1	12 months follow-up: **DRB1, DQB1, DPB1 together** **No effect overall** (immunoreaction); ns **No effect normal risk** (immunoreaction); ns **Beneficial high risk** (immunoreaction); p = 0.02 1-4 matches 17% immunoreaction (n = 23); 0 matches 50% immunoreaction (n = 28) **Separate analysis of the loci for overall, normal, high risk** **DRB1 no effect** (immunoreaction), although trend for benefit in high risk group **DQB1 no effect** (immunoreaction), although trend for benefit in high risk group **DPB1 beneficial high risk** (immunoreaction): p = 0.01 1-2 matches 7% immunoreaction (n = 14); 0 matches 46% immunoreaction (n = 37) NB: other factors of influence are not mentioned or corrected for.
Munkhbat et al, 1999	A, B	12 months follow-up: **A, B** **Beneficial overall**; p = 0.03 1-4 matches 19% immunoreaction (n = 42); 0 matches 41% immunoreaction (n = 37) **Beneficial high risk**; p = 0.008 1-4 matches 17% immunoreaction (n = 24); 0 matches 52% immunoreaction (n = 25) **A separate** **Beneficial overall**; p = 0.001 1-2 matches ± 12% immunoreaction (n = 26); 0 matches ± 39% immunoreaction (n = 54) **Beneficial high risk**; p = 0.02 1-2 matches ± 14% immunoreaction (n = 16); 0 matches ± 48% immunoreaction (n = 34) **B separate** **No effect overall**; p = 0.60 1-2 matches ± 25% immunoreaction (n = 24); 0 matches ± 31% immunoreaction (n = 55) **No effect high risk**; p = 0.17 1-2 matches ± 17% immunoreaction (n = 12); 0 matches ± 40% immunoreaction (n = 37) NB: other factors of influence are not mentioned or corrected for.
Volker-Dieben et al, 2000	A, B / *DR*	**A, B** **Beneficial normal risk** (immunoreaction); p = 0.05 2-4 matches 9% immunoreaction (n = 480); 0-1 matches 13% immunoreaction (n = 349) **Beneficial high risk** (immunoreaction); p < 0.001 2-4 matches 25% immunoreaction (n = 642); 0-1 matches 42% immunoreaction (n = 207) **Corrected for vascularization status: beneficial: p = 0.01** ***DR*** **No effect normal risk** (immunoreaction); p = 0.14 2 matches 7% immunoreaction (n = 69); 0-1 matches 12% immunoreaction (n = 209) **Beneficial high risk** (immunoreaction); p = 0.02 2 matches 15% immunoreaction (n = 66); 0-1 matches 29% immunoreaction (n = 214) **Corrected for vascularization status: beneficial: p = 0.03** Confounding factors (having influence on outcome, but not corrected for): graft size, p = 0.002; organ culture, p = 0.001; gender, p = 0.01; HLA-A,B and DR

Khaireddin et al, 2003 (Update on study Hoffmann et al., 1994)	A, B, DR	**Beneficial normal risk** (immunoreaction); p = 0.04 2-6 matches 12% immunoreaction (n = 249); 0-1 matches 26% immunoreaction (n = 46) **Beneficial high risk** (immunoreaction); p < 0.001 2-6 matches 41% immunoreaction (n = 130); 0-1 matches 92% immunoreaction (n = 13) NB 1: analysis at split level added no advantage NB 2: other factors of influence are not mentioned or corrected for.
Bartels et al., 2003	A, B	**Split level analysis** **Beneficial high risk** (immunological graft failure); p = 0.002 3-4 matches ± 15% immunological graft failure (n = 216); 0-2 matches ± 31% immunological graft failure (n = 87) NB: the beneficial effect remained significant with an odds-ratio of 0.41, after multivariate analysis correcting regrafts and indication for transplantation. **Beneficial high risk** (overall graft failure); p = 0.04
Reinhard et al, 2004a	A, B, DR	Four year follow-up **A, B, DR** **Beneficial normal risk** (immunoreaction); p = 0.048 4-6 matches 8% immunoreaction (n = 66); 0-3 matches 28% immunoreaction (n = 352) **Beneficial normal risk** (rejection-free clear graft); p = 0.03 4-6 matches 8% no rejection-free/clear graft (n = 66); 0-3 matches 34% no rejection-free/clear graft (n = 352) Confounding factors (having influence on outcome, but not corrected for): organ storage time, p = 0.03.
Böhringer et al., 2004	A, B, DR	**Split level analysis A, B (triplet-string matching)** **Beneficial normal risk** (immunoreaction); p < 0.05 <13 triplet mismatches 15% immunoreaction (n = 147); > 13 triplet mismatches 24% immunoreaction (n = 398) NB 1: groups differed significantly for DR matches (p = 0.02). NB 2: Cox multivariate regression analysis was used, yet it is unknown which confounding factors were included. **Broad level analysis A, B** **No effect normal risk** (immunoreaction); p = 0.08 3-4 matches 8% immunoreaction (n = 57); 0-2 matches 24% immunoreaction (n = 488) NB: groups differed significantly for DR matches (p < 0.01). **DR** Analysis not performed due to too small group size.
Minor antigens		
Inoue et al., 2000b	H-Y	**No effect normal risk** (immunoreaction); p = 0.71 Matched (male-male) ± 25% immunoreaction (n = 175); Mismatched (male-female) ± 27% immunoreaction (n = 53) **No effect high risk** (immunoreaction); p = 0.70 Matched (male-male) ± 40% immunoreaction (n = 127); Mismatched (male-female) ± 40% immunoreaction (n = 41) NB: no assessment or correction for possible confounding effect of HLA type.
Reinhard et al, 2004a	H-Y	**No effect normal risk** (immunoreaction); ns Matched (male-male) (n = 291); Mismatched (male-female) (n = 418) No other data mentioned except relative risk of 1.1. NB: no data provided on possible confounding effect of HLA type.
Böhringer et al., 2006 (Reinhards group)	H-Y, HA-3	**H-Y** **Beneficial overall** (immunoreaction); p = 0.02 Matched 12% immunoreaction (n = 148); Mismatched 23% immunoreaction (n = 81) **H-Y** **No effect overall** (immunoreaction); p = 0.52 Matched 15% immunoreaction (n = 148); Mismatched 27% immunoreaction (n = 81) NB 1: p-values are of multivariate analysis adjusting for risk group, HLA-A1 mismatch and patient age. NB 2: no assessment or correction for possible confounding effect of HLA type (although data was available).

Reports on the systemic use of immunomodulatory biologics for the prophylaxis and treatment of human corneal graft rejection are relatively few. In a small study reported in the German-language literature, basiliximab (a chimaeric monoclonal antibody with specificity for CD25) in combination with corticosteroids was found to be moderately effective for the prophylaxis of corneal graft rejection.[(Birnbaum et al., 2008)] Alemtuzumab, originally known as Campath-1 (a humanized monoclonal antibody with specificity for CD52) was used systemically to reverse corticosteroid-resistant corneal allograft rejection in several recipients, with some success.[(Dick et al., 2000; Newman et al., 1995)] However, administration of these specific biologics is not without risk in either the short or long term and their side-effects profiles, and need for continuous monitoring by non-ophthalmologists together with their limited efficacy, probably explain why neither has as yet found widespread favour as an immunosuppressant for keratoplasty.

In recent times, the use of topical or subconjunctival administration of biologics to target the cornea has generated some interest. Large biologics of the size of intact antibody molecules will not pass across the human ocular surface over any reasonable time-frame,[(Allansmith et al., 1979)] although it is possible to enhance the penetration of engineered small antibody fragments into the cornea.[(Thiel et al., 2002, 2013)] However, topical administration of a biologic for the successful prophylaxis or treatment of human corneal graft rejection has not yet been achieved. Subconjunctival administration is a well-established method for producing a depot of a drug that may influence disease processes in the cornea and anterior segment. The application of anti-vascular endothelial growth factor (VEGF) biologics delivered by subconjunctival injection is actively being examined as a means to limit corneal neo-vascularization in humans, especially in cases of trauma or infection. Aside from reducing the direct consequences of vascularization upon vision, the rationale is to increase the chances of success of a corneal graft (should one subsequently be required), given that neovascularization is a well-established risk factor for rejection-mediated corneal graft failure.[(Coster and Williams, 2005)]

Cursiefen and co-workers have demonstrated the importance of lymph vessels in corneal transplantation. Immunohistochemistry was applied to human corneas to determine the presence of LYVE-1 and podoplanin- (two markers of lymphangiogenesis) positive vessels. About 8% of vessels in vascularized corneas were lymph vessels. There was a strong correlation between the presence of lymphatic vessels and hemangiogenesis.[(Cursiefen et al., 2002)] Anti-angiogenesis treatments that decreased both blood and lymph vessel ingrowth were able to restore the immune privilege. VEGF inhibitors such as bevacizumab were found to effectively block corneal angiogenesis as well as lymphangio-genesis.[(Bock et al., 2007)] Blocking lymph- and hemangiogenesis with an inhibitor of VEGF (VEGF Trap (R1R2)) greatly improved corneal transplant survival (Cursiefen et al., 2004). Similarly, blocking VEGF-A or VEGFR-3 post-transplantationally resulted in less vessels and subsequently less APC trafficking, ending in improved graft survival in an experimental model.[(Bachmann et al., 2008; Chen et al., 2004; Niederkorn and Larkin, 2010)] A recent randomized, placebo-controlled clinical trial of three subconjunctival injections of 2.5 mg bevacizumab for new-onset corneal neovascularization demonstrated a significant reduction in the area of corneal vessels in the treated group, compared with an increase in the area of new

vessels in the control group, at 3 months after initiation of treatment (Petsoglou et al., 2013). The intervention was reportedly well tolerated, with no major safety concerns, and could as such provide a good measure to reduce the risk of corneal graft rejection. Another approach may be the inhibition of the VEGF-receptor related kinases, such as VEGFR-tyrosine kinase. Application of a VEGFR-tyrosine kinase inhibitor led to improved survival of corneal transplants in a murine model.(Hos et al., 2008)

As antigens from the AC drain to preauricular and submandibular lymph nodes,(Camelo et al., 2005) local lymphadenectomy in mice helped to prevent antigens from reaching the local lymphoid tissue, and prolonged corneal graft survival.(Plskova et al., 2004; Yamagami and Dana, 2001) One may also attack the lymph vessels: CD11b+ macrophages express LYVE-1 and Prox-1 under inflamed conditions in the murine cornea, and these cells were able to form vessel-like structures in vivo. Blocking such cells may help in preventing (lymph)angiogenesis.(Maruyama et al., 2005)

Depletion of antigen-presenting cells may be an alternative for HLA Class II matching. This may effectively block antigen-presentation by APC depletion. It has been shown that blocking corneal and conjunctival antigen-presenting cells by local depletion with clodronate-containing liposomes completely inhibited graft rejection in a murine model.(Slegers et al., 2000) A problem with this approach is the simultaneous blocking of local innate immune protection against pathogens, such as Acanthamoeba.(Van Klink et al., 1996) On the other hand, this approach is inherent to using grafts that have been kept in culture for a while: reduction of HLA Class II expression was found in corneas stored for two weeks in organ culture.(Al-Fakih et al., 2012; Mayer et al., 2007) However, time and type of storage may influence the quality of the graft. A disadvantage of storing corneas for a longer time is that it may adversely affect reepithelialization, even if storage in Optisol-GS lasted less than 14 day.(Lam et al., 2013)

Other immunological treatments such as blocking of CD4+ T cells will, unless topically applicable, likely result in significant systemic immunosuppression, which is therefore unlikely to be used clinically as preventive treatment in clinical corneal transplantation. Many different immunomodulatory biologics have been examined for their efficacy in prolonging corneal graft survival in experimental animals. Because mice and rats have open lymphatics in the peritoneal cavity, intraperitoneal injection has been the favoured means of delivery in these species, but such administration is clearly not applicable for humans. Furthermore, the immune systems of mice and men differ substantially in many respects.(Mestas and Hughes, 2004) Ophthalmologists have naturally had reservations about administering any of these potent immunosuppressants systemically to their patients with corneal grafts, without a great deal more evidence of efficacy. Corneal graft rejection is a so-called “orphan disease”,(Aronson, 2006) so that large pharmaceutical companies are unlikely to expend resources on the discovery of new biologic drugs for this relatively rare condition. The role of biologics in the future is thus likely to be restricted to agents that have already been licensed for use in other, more common diseases. In such instances, the safety profile at least will already be reasonably well-established, as was the case with bevacizumab, licensed initially for use in colon cancer.

5.2. Potential of gene therapy to prolong human corneal graft survival

The potential of gene therapy to modulate corneal diseases in general and corneal graft rejection in particular has been extensively - even exhaustively - studied and reviewed over the past 15 years. (Borras, 2003; George et al., 2000; Jun and Larkin, 2003; Kampik et al., 2012; Mohan et al., 2005; Parker et al., 2009; Qazi and Hamrah, 2013; Ritter et al., 2013; Williams et al., 2004) The usual approach is to modify the donor corneal endothelium ex vivo, prior to transplantation, with a transgene designed to influence the afferent or efferent arm of the allograft response. This is a clinically-relevant scenario, as donor corneas are preserved in the eye bank for varying periods prior to release for keratoplasty.

Increasing the expression of the factors that mediate immune privilege by gene transfer of immunomodulatory molecules such as TNF-receptor, TGF-beta, IL-10, IL-12, and NGF (Nerve Growth Factor), may increase or restore corneal immune privilege and prolong graft survival. This has been investigated in several animal models;(Beutelspacher et al., 2006; Comer et al., 2002; Gong et al., 2007; Klebe et al., 2001a, 2005; Rayner et al., 2001; Ritter et al., 2007) however, most factors were not able to prolong corneal survival when applied alone, with the exception of NGF.(Gong et al., 2007)

The choice of the animal model is especially important in studying ex vivo gene transfer to corneal endothelium, because unlike the situation in humans and larger mammals, the endothelial cells of small rodents can replicate.(Tuft et al., 1986) A positive outcome in a small animal model thus demands confirmation in a larger, preferably outbred animal model, several of which are available.(Klebe et al., 2001b; Nicholls et al., 2012) Despite a number of partial successes, overall outcomes have thus far been somewhat underwhelming. Indefinite and biologically-significant, long-term prolongation of corneal graft survival has seldom been achieved in the majority of animals tested, a likely requirement of the gene technology regulators before permission would be forthcoming for any clinical trial. Not surprisingly, then, ex vivo gene therapy directed at a donor cornea prior to clinical transplantation, with the ultimate goal of prolonging the survival of that graft, has not been reported. This is in stark contrast to success in the use of gene therapy for inherited monogenic retinal disorders such as Leber's congenital amaurosis.(Bainbridge et al., 2008; Maguire et al., 2008) One likely reason lies in the remarkable degree of redundancy that is evident in the immune response to an allograft. Possibly not one transgene will prove sufficiently broad-acting enough on its own to modulate the immune response to a corneal graft completely. Whether a combinatorial approach will be more successful has yet to be thoroughly tested. Topical, rather than systemic therapies, and lamellar keratoplasty, are the current approaches that are being applied to reduce corneal allograft rejection. The most successful future therapies would be those that target more than one pathway.

5.3. Cellular suppression

A potential new approach to prevent or treat rejection may be found in the use of mesenchymal stem cells. Bone marrow-derived mesenchymal stem cells (MSCs) are non-hematopoietic cells, that are capable of a range of anti-inflammatory functions, and can reduce corneal rejections in mouse and rat transplantation models,(Lan et al., 2012; Oh et al., 2012) reviewed by Li and Zhao (2014).(Li and Zhao, 2014)

They have been found to stimulate tissue repair[(Lan et al., 2012)] by increasing the expression of anti-inflammatory cytokines such as TGF-beta and IL-1Ra. Intravenously injected MSCs home to damaged (inflamed) tissues, including a cornea that has undergone a corneal transplant.[(Omoto et al., 2014)] When injected at the time of a corneal transplantation in mice, these cells are able to bring down the number of APCs in the cornea and draining lymph nodes, as well as the number of induced Th1 cells, while improving graft survival. Earlier, Oh et al.[(Oh et al., 2012)] reported that injection of MSCs at the time of corneal transplantation in mice would reduce rejection. On day 7 post-transplantation, the number of dendritic cells and macrophages was reduced in corneas of mice that had received MSCs. The injection of MSCs reduced not only the aspecific early inflammation induced by surgery but also the allo-specific response observed on day 28 post transplantation. The production of a soluble factor, tumor necrosis factor-alfa stimulated gene/protein 6 (TSG6) was involved and infusion of recombinant TSG6 was also capable of reducing rejection rate, by reducing the local corneal immune response. Using an immunosuppressive factor would make the use of cells redundant. However, if cells would be more efficient, it raises the question whether the MSCs need to be compatible with the cornea donor. Obtaining donor-derived human MSCs or cells derived from other sources such as the umbilical cord might be feasible for human use.

6. Discussion

Corneal transplantation is considered one of the most successful form of transplantation in humans. Long-term survival rates in low-risk non HLA-matched patients are higher than those seen in HLA-matched solid organ transplants.[(Cecka, 2010; Claas et al., 2005; Coster and Williams, 2005; Gondos et al., 2013; Ing et al., 1998; Inoue et al., 2000a; Thompson et al., 2003; Williams et al., 1997)] A prospective study on PKPs for normal risk cases with a median follow-up of 18 months showed rejection episodes in 11% of the grafts in the first 18 months. Risk factors for rejection were atopic dermatitis, clinically-manifest tear insufficiency and short storage of the graft.[(Kuchle et al., 2002)] This shows that in normal risk cases, graft rejection can occur, but is not common. Next to penetrating keratoplasty, other allografting methods such as ALK and deep-ALK for anterior corneal disease are more frequently applied, improving the survival rates even further by leaving the endothelium intact. Lamellar transplants are usually performed in diseases where vascularization is absent. A survival rate of 99% over 9 years has been reported in deep-ALK performed for stromal disease.[(Sarnicola et al., 2012)] The group of 806 eyes consisted of keratoconus (74%), postherpetic keratitis scarring (15%), and other stromal opacities. This excellent success is mainly attributed to the dimin- ished endothelial cell loss for deep-ALK compared to PK.[(Borderie et al., 2012; Kubaloglu et al., 2012)] However, one should take into account that ALK is especially used in keratoconus, a disease that consists of thinning and malformation of the cornea, usually without inflammation or blood vessel formation, which would also have excellent survival in perforating grafts. PKPs performed in keratoconus are hardly ever rejected, so it is logical that laminar grafts for this disease are also not prone to rejection.[(Thompson et al., 2003)] The influence of ALKs on the overall corneal survival rates to date

is minimal as 81-94% of the corneal allografts still replace the endothelium.[Konijn-Janssen et al., 2011; Lichtinger et al., 2012; Williams et al., 2012] In the posterior transplants, one either replaces Descemet's membrane with a new Descemet membrane together with the endothelial cells, or with a combination of Descemet's with endothelium and an additional thin layer of corneal stroma.[Melles et al., 2000, 2008] In both cases, the target of endothelial rejections is the donor tissue; although long-term results remain to be determined, the first prospective studies are reporting 5-year survival rates around 72.5% for DLEK (Deep Lamellar Endothelial Keratoplasty),[Mashor et al., 2010] and 89% for DSEK (Descemet's Stripping Endothelial Keratoplasty),[Anshu et al., 2012a] while the 5-year survival for full thickness grafts for Fuchs' dystrophy is 83-87%.[Cheng et al., 2013; Fasolo et al., 2011; Thompson et al., 2003] Although these grafts are especially performed for corneas with primary or secondary Fuchs' dystrophy, a fast loss of endothelial cells may also occur following the transplantation, independent of the technique applied.[Chan et al., 2012] A study that compared DMEK (Descemet's Membrane Endothelial Keratoplasty, n = 141), DSEK (n = 598) and PK (n = 30) in patients with similar demographics and indications for surgery, showed a 2-year survival rate of 99% for DMEM, 88% for DSEK, and 82% for PK.[Anshu et al., 2012b] However, recent data from the Australian Corneal Graft Registry Study, comparing the graft survival of 1643 lamellar and 9875 penetrating keratoplasties between 1996 and 2013, showed that the survival of DALKs (deep anterior lamellar keratoplasty) and endokeratoplasties is worse than the survival of penetrating keratoplasties performed for the same indications (mostly keratoconus for DALK, and mostly Fuchs' dystrophy or pseudophakic bullous keratopathy for endokeratoplasties).[Coster et al., 2014] When focusing on irreversible rejection as the reason for graft failure, PK was the least favourable with 30% (635/2094) of the failures due to irreversible rejection, compared to only 2% (1/53) for DALK and 12% (37/318) for endokeratoplasty. Rejection can thus still occur, although at a lower rate, but the current results show a lot of failures due to other causes. When focusing on DALK for keratoconus, the rejection rate for anterior lamellae was very low. Overall survival was better for DALK at three years, but when early failures were excluded, survival was similar.[Jones et al., 2009]

Despite (technical) advances, immunological rejection remains a major reason for graft failure in all types of grafts, both in low- and high-risk patients. Strikingly, for the high-risk patients, the prognosis of corneal graft survival is similar to that of solid organ transplants,[Cecka, 2010; Claas et al., 2005; Thompson et al., 2003; Williams et al., 1997] and patients may receive systemic treatments to prevent their corneal graft from being rejected.[Joseph et al., 2007; Nguyen et al., 2010] Considering this, it seems that transplanted corneas are ‘forgotten grafts’ with regard to the number of adequate studies investigating the reasons leading to corneal allograft rejections.[George and Larkin, 2004] Furthermore, notwithstanding that in corneal allografting, HLA typing and proper allocation is generally assumed to be unnecessary, most studies show that outcomes in high risk cases are better for increasingly-matched grafts and best for those grafts which have no HLA differences with the recipient at all. Obviously, finding a good match can be difficult due to the high polymorphism of the HLA system. In solid organ transplantation where the benefit of HLA matching is evident, there is not always time to wait for a completely compatible donor, as waiting might prove fatal to the patient. Corneal transplantation however, is performed to

restore or improve sight and not to save life, and with the exception of emergency transplants, this often provides the valuable time needed to find a compatible donor. With longer cornea storage times, possibly up to one year,[(He et al., 2012)] even more time will be available for proper matching. Worldwide, a great shortage exists in the number of grafts available, and this is even the case in countries with a long tradition of cornea banking. With the increasing number of elderly patients, more corneas will be necessary, and the lower the number of necessary re-grafts, the more patients can be helped. Therefore, it would be a waste not to invest in studies to determine properly the benefit of matching corneal allografts for HLA minors in addition to HLA Class I antigens.

From an immunological point of view, studies on the effect of HLA matching should ideally take immunological rejection, not necessary leading to graft failure, as end point. This would help to better differentiate between the causes of graft failure, and thus help to develop preventive measures. Comparing this graft rejection-free survival time is more appropriate than using graft failure-free survival and might give more adequate results, even more so when DNA-based HLA matching at broad or split-level is used instead of the less accurate serological matching. A system identifying acceptable HLA mismatches and only matching for the most relevant and significant HLA alleles, could be a valuable, and time-and money-saving approach.[(Claas et al., 2005, 2009; Opelz and Dohler, 2007)] Currently, the FANCY trial (Functional Antigen Matching in Corneal Transplantation, NCT00810472), running from 2009, is investigating the results of HLA matching in a blind randomized study. Patients are being matched using the HLAMatchmaker algorithm, identifying acceptable mismatches for sensitized patients, and the end-point is first endothelial graft rejection. In the UK, the CTFS II/Corneal Transplant follow-up Study II is a multi-center study that was commenced in 1998 (ISRCTN 25094892). In this study, a waiting list of HLA-typed patients was established, to maximize the chances of patients getting a matched graft. This study should provide information on the role of HLA-DR in graft survival. Efforts to improve corneal graft rejection-free survival need also take into account the effect of minor H antigens, as in mice these seem to be able to negate the MHC matching effect. The advantage of better matching is a better graft survival without side effects, which is not going to be the case for medical interventions.

An interesting question is whether the genetic make-up of the recipient influences the rate of rejection: the CTFSII study in the UK also investigates the role of different gene polymorphisms that may influence the outcome of cornea transplants. The genotypes of different cytokine genes were determined in 384 patients undergoing a full-thickness transplant, and related to the occurrence of rejection during the first three years after transplantation. Specific haplotypes of the TNFalfa gene were either associated with a reduced or an increased risk of rejection.[(Winton et al., 2014a)] Similar results were reported for polymorphisms in VEGF-A and the IL-17F gene, which together with a specific TNFalfa polymorphism seem to make up a “high inflammatory haplotype”, that leads to a higher chance of rejection.[(Winton et al., 2014b)] It is fascinating that these genes are located on chromosome 6. It does however mean, that a determination of the genetic make-up of the recipient, independent of

the corneal recipient status, may be used to assess the risk of rejection, and therefore the need to perform HLA matching or not.

The cost-effectiveness of HLA matching for keratoplasties also plays a role in the decision to perform HLA matching. Baumler et al. performed a retrospective analysis and used information that typing donor and recipient cost 1200 euro, and came to the conclusion that proper matching would give a prolonged rejection/failure-free survival of more than 1000 days.[(Baumler et al., 2014)] The incremental cost of HLA matching would than range from 2.10 euro to 6.71 euro per additional day of graft survival, which is very acceptable given the high cost of regrafting and the shortage of corneal donors worldwide.

7. Conclusion and future directions

Despite controversial results of the effect of HLA matching on corneal allograft survival in older studies, we should not ignore that recent studies, using more accurate typing methods, provide consistent evidence that HLA matching is beneficial to corneal allograft survival in general and even more in high-risk allografts. The corneal transplantation field can take advantage of the results coming from solid organ HLA matching, as once the immune privilege has been compromised, the same immunological mechanisms apply. Obviously, and especially from a patient's view, successful prevention of immune rejection, rather than immune suppression by post-transplantation treatments or immune-modifying treatments, such as gene therapy, remains the best primary approach. Combining HLA matching with other preventive immunological therapies, thus targeting multiple pathways at the same time, may even further enhance long-term corneal graft survival.

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CHAPTER 5

A Comparison of HLA Genotype with Inflammation in Uveal Melanoma

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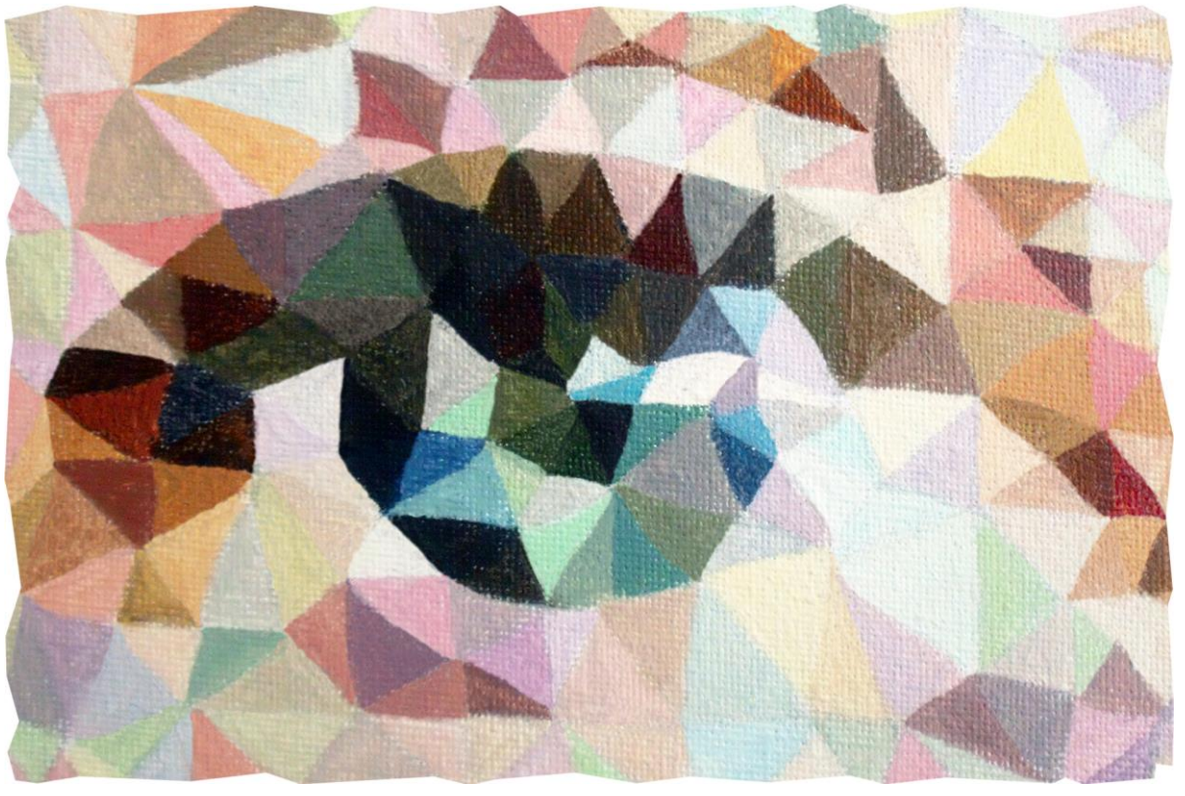

Abstract

Purpose: Human leukocyte antigen (HLA) polymorphisms have been associated with the development of autoimmune diseases. In uveal melanoma, a high expression of HLA classes I and II, and infiltration with lymphocytes and macrophages are associated with a bad prognosis. Inflammation has an important role in this malignancy. The goal of our study was to determine whether specific HLA alleles are associated with increased inflammation.

Methods: Records were analyzed of 45 patients who underwent enucleation for uveal melanoma. HLA typing, tumor HLA expression and tumor macrophage infiltration were determined in each case.

Results: Before correction for multiple testing, macrophage infiltration was less in HLA-A2 positive patients. Patients with HLA-DR6 had a higher tumor cell expression of HLA-DR. After correction for the number of analyses, no associations remained statistically significant.

Conclusion: The results before correction suggest that the HLA genotype may influence inflammation as indicated by HLA expression and macrophage infiltration in uveal melanoma. However, after correction this association did not prove significant.

Introduction

Mechanisms by which tumor cells evade the immune system have an important role in tumor growth, as emphasized by the cancer immunosurveillance theory.[1] Among these escape mechanisms, alterations in the expression of classical and non-classical human leukocyte antigen (HLA) classes I and II antigens on tumor cells are of a particular interest. These antigens have a crucial role in the induction of specific immune responses, and can modulate the interactions of natural killer (NK) cells and T cell subpopulations with their target cells.[2–4] The clinical relevance of HLA class I expression in oncology is evident from the finding that a reduced expression often correlates with a worse prognosis,[5–10] while the relevance of HLA class II expression in cancer remains ambivalent.[1,11,12]

Unlike most tumors, in uveal melanoma a high HLA class I expression is considered to be a bad prognostic sign.[13–16] This observation may be explained by the role of NK cells. Tumors with high HLA class I expression are more resistant to destruction by NK cells, as these cells specifically kill cells that lack certain HLA class I antigens.[16–18] Metastases of primary uveal melanoma show a high HLA-A and B expression,[19] thus suggesting evasion of NK cells by the tumor is necessary in the development of metastases. In uveal melanoma, a high expression of HLA class II also is related to a bad prognosis.[15]

A small, but growing number of studies have indicated that, apart from HLA expression, HLA polymorphisms mediate susceptibility to certain neoplastic diseases.[20,21] In several uveal melanoma studies an increased incidence of HLA-A32 has been found,[22–25] and another study revealed that patients with HLA-B40 died more often from metastases.[26] Nevertheless, a study on 235 cases could not confirm this association between HLA- B40 and metastasis; however, the study did find an association between HLA-B44 and metastasis before correction. Therefore, a role for HLA genotype in prognosis is not excluded.[27]

Other important immunological parameters associated with prognosis in uveal melanoma are lymphocyte[28] and macrophage[29] infiltration. High numbers of tumor-infiltrating $CD68^+$ and $CD163^+$ macrophages are associated with an unfavorable prognosis,[29–31] and $CD68^+$ macrophages have been associated with increased HLA classes I and II expression as well.[32] Several specific associations are known between HLA antigens and ocular diseases. It may well be that certain local immune responses may protect against the development of uveal melanoma by providing a local immunosurveillance system, for example against aberrant melanocytes. Choroidal auto-immune responses are noticed in birdshot chorioretinopathy (BCR), which is characterized by multiple hypopigmented chorioretinal lesions, and is associated with HLA-A29.[33] In Vogt-Koyanagi-Harada syndrome (VKH), a bilateral, chronic, diffuse panuveitis with late stage depigmentation of the fundus occurs, with a genetic association with primarily HLA-DR4.[34] Finally, uveitis in relation to autoimmune diseases has been related to HLA-B27.[35,36] Therefore, we investigated whether the most common as well as these specific HLA genotypes are related to the level of HLA expression on tumor cells and intra-tumoral macrophage infiltration, which can be regarded as parameters of inflammation in uveal melanoma.

Material and Methods

Study Population and Data Set

Records were analyzed of 50 patients with uveal melanoma who underwent enucleation between 1999 and 2004 at the Leiden University Medical Center in the Netherlands.

Table 1. Distribution of Sex, Age, and Pathological TNM Classification for Each Group

	Original Group* (*n* = 50)	**HLA Expression (*n* = 45)**	**Macrophage Infiltration (*n* = 38)**
Males (mean age)	23 (60 y)	22 (59 y)	19 (60 y)
Females (mean age)	27 (61 y)	23 (62 y)	19 (62 y)
Stage I	10%	9%	5%
Stage IIA	22%	22%	21%
Stage IIB	28%	24%	26%
Stage IIIA	34%	38%	40%
Stage IIIB	6%	7%	8%
Stage IV	0%	0%	0%

*Maat et al.[32]

For 45 patients, the HLA genotype was available. Information on the presence or absence of one chromosome 3 in the tumor was available for all 45 cases.[37] The research protocol followed the tenets of the Declaration of Helsinki (World Medical Association Declaration of Helsinki 1964, ethical principles for medical research involving human subjects). All patients signed an informed consent form.

Collection of Specific Data

The specific HLA genotype of each patient had been determined previously[27] and was retrieved from the patients' chart. All results of DNA-based techniques were transformed to the serological classification to obtain unified data, usable for statistical analysis.

Immunostaining of tumor cells of these patients for expression of HLA-A, HLA-B and HLA-DR had been performed previously and described by Maat et al.,[32] using the monoclonal antibodies (mAbs) HCA2, which stains exclusively HLA-A, and HC10, which binds to HLA-B and HLA-C.[38,39] Both antibodies were obtained from the Dutch Cancer Institute (Amsterdam, The Netherlands). For HLA-DR staining Tal.1B5 was used, obtained from DakoCytomation (Glostrup, Denmark).

Phenotypic characterization of macrophages and digital counting were performed previously by Bronkhorst et al.[31] using immunofluorescence double-staining with mAbs against CD68 and CD163. The amount of positive staining was calculated as pixels per mm^2.

Comparison of Data

To minimize loss of significance after correction for the number of tests performed, only common and well-defined HLA alleles with the highest frequency in 2440 healthy Dutch blood donors were selected for statistical analysis.[40] In detail, we selected three alleles for HLA-A (A1, A2, A3) and the two major grouping alleles for HLA-B (Bw4, Bw6), while for HLA-DR two alleles (DR4 associated with VKH, DR6) were used. Additionally, we selected alleles associated previously with uveal melanoma and bad prognosis (HLA-B40 and -B44), and two alleles associated with ocular autoimmune diseases (HLA-A29 and HLA-B27).

All statistical analyses were performed with a statistical software program (SPSS for Microsoft Windows, version 17.0.2; SPSS Inc., Chicago, IL). The hypothesis was tested with the Mann-Whitney U test (Wilcoxon rank test) for non-normal distribution for each specific HLA allele. For the comparison of each HLA allele with chromosome 3 status, the v^2 test was used. Statistical significance was assumed for a P value <0.05. Correction for multiple testing was performed according to the Bonferroni correction for the number of alleles (n = 8) tested.

Results

General Results

The HLA genotype of 45 patients was collected. Tissue specimens for macrophage staining were available for 38 patients. Tumor staging according to the pathologic TNM staging of the 7th edition of the American Joint Committee on Cancer International Union on Cancer (AJCC-UICC),[41] and sex and age distribution of these patients is shown in Table 1. The frequencies of the studied HLA alleles in patients with uveal melanoma were compared to those of healthy Dutch blood donors and showed a comparable distribution (Table 2).

The VKH-associated HLA-A29 was present only in four cases, while the autoimmune uveitis-related HLA allele B27 was not present in the population being studied. HLA-B40, associated previously with bad prognosis in uveal melanoma, is now split into B60 and B61. Only five patients carried B60, while no patients were positive for HLA-B61. Therefore, the relationship between these alleles and the degree of HLA expression or macrophage infiltration was not determined.

Expression of HLA I and II Molecules

Sections of uveal melanoma stained with HCA2 (HLA-A), HC10 (HLA-B and HLA-C) and Tal.1B5 (HLA-DR) had been analyzed previously. Figure 1 shows an example of a section stained with HCA2. The percentage of cells that reacted positively with the anti-HLA class I antibodies HC10 and HCA2 varied widely, with a mean of 43% for HCA2 (SD 29%) and 37% for HC10 (SD 31%). The mean percentage of HLA-DR-positive cells was 20% (SD 23%).

Staining for HCA2 correlated positively with HC10 staining ($P < 0.001$). Staining for Tal.1B5 was correlated with HCA2 and HC10 staining ($P = 0.01$ and $P = 0.005$, respectively, Spearman test).

Table 2. Distribution of the Selected HLA Alleles in the Study Group and a Dutch Blood Donor Database

	Study Group (*n* = 45)		Dutch Blood Donors (*n* = 2440)*		
HLA Allele	**Positive (*n*)**	**Positive (%)**	**Positive (n)**	**Positive (%)**	***P* Value**
A1	14	31	747	31	0.94
A2	24	53	1284	53	0.93
A3	16	36	700	29	0.31
A29	4	9	119	5	0.22
B27	0	0	157	6	0.11†
B44	10	22	586	24	0.78
B60 (B40)	5	11	361	15	0.49
B61 (B40)	0	0	73	3	0.64
Bw4	23	51	1346	55	0.59
Bw6	38	84	2143	88	0.49
DR4	13	29	679	28	0.88
DR6	14	31	796	34	0.83

P values for each allele are given (χ^2 test).

*Schipper et al.[40]

†Fisher's exact test was used due to zero positive cases in the study group.

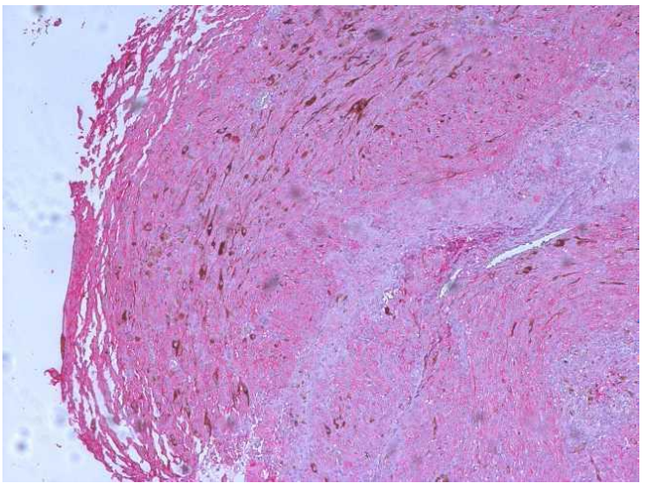

Figure 1. Immunohistochemical staining with the antibody HCA2 for HLA class I expression in uveal melanoma. 100x magnification.

Expression of HLA I and II Molecules

Sections of uveal melanoma stained with HCA2 (HLA-A), HC10 (HLA-B and HLA-C) and Tal.1B5 (HLA-DR) had been analyzed previously. Figure 1 shows an example of a section stained with HCA2. The percentage of cells that reacted positively with the anti-HLA class I antibodies HC10 and HCA2 varied

widely, with a mean of 43% for HCA2 (SD 29%) and 37% for HC10 (SD 31%). The mean percentage of HLA-DR-positive cells was 20% (SD 23%).

Staining for HCA2 correlated positively with HC10 staining ($P < 0.001$). Staining for Tal.1B5 was correlated with HCA2 and HC10 staining ($P = 0.01$ and $P = 0.005$, respectively, Spearman test).

HLA Allele versus HLA Expression

As in autoimmune diseases some HLA alleles are associated with increased inflammation, we wondered if this were the case in uveal melanoma. Therefore, we compared staining of uveal melanoma cells for HCA2, HC10 and Tal.1B5 with the presence of frequently-occurring HLA alleles in 45 patients, as well as with alleles associated previously with prognosis in uveal melanoma, and alleles well-known for their association with ocular auto-immune diseases. HLA-A29 was present in only four cases, HLA-B27 in none, and HLA-B60 in five, and comparisons, therefore, were not feasible. HLA-B44 was present in 10 cases, but showed no correlation with HLA expression.

HLA-DR4 was associated almost significantly with a decreased expression of HLA-DR ($P = 0.06$), while HLA-DR6 was associated with a higher HLA-DR expression ($P = 0.04$). Significance was lost after correction for the total number of tests. An overview of HLA alleles versus HLA expression is shown in Table 3.

Macrophage Infiltration

Infiltration of macrophages had been assessed previously using immunofluorescence staining on paraffin-embedded sections of uveal melanomas from 38 patients. An example of this staining is shown in Figure 2. The presence of positive cells was measured as total amount of staining per section. The area of $CD163^{+}CD68^{+}$ double-staining associated with the area of $CD68^{+}$ (Spearman test, $P < 0.001$).

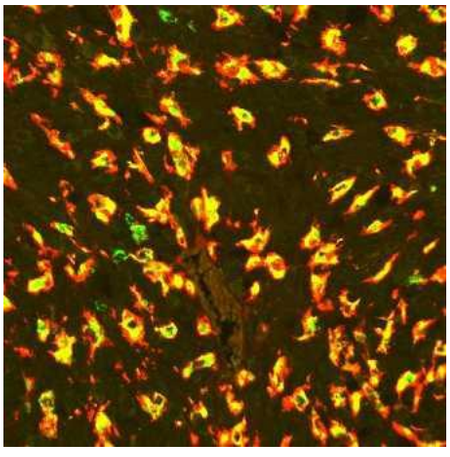

Figure 2. Immunofluorescence staining of macrophages in uveal melanoma using two antibodies directed against CD68 or CD163. Fluorescent staining of CD68 is shown as green and of CD163 as red. Overlay of both stainings showing double-positive cells are visualized in yellow. 250x magnification.

A comparison of $CD68^+$ cells with HLA expression showed that an increased density of macrophages was associated with an increased number of cells positive for staining with mAbs HCA2, HC10, and Tal1.B5 (respectively, $P = 0.02$, $P < 0.001$, and $P < 0.001$, Spearman test).

HLA Allele versus Macrophage Infiltration

The most common HLA alleles together with HLA B44 were compared with macrophage infiltration and phenotype. Of the class I HLA alleles, only where the HLA-A2 allele was present, less $CD68^+$staining ($P = 0.03$) as well as less $CD68^+CD163^+$ staining ($P = 0.02$) was observed. With regard to HLA-DR4 and -DR6, there was no increased or decreased macrophage infiltration compared to other alleles. When corrected for the total number of tests, significance was lost for all associations between HLA genotype and macrophage infiltration. An overview of the distribution of macrophage infiltration per HLA allele is shown in Figures 3 and 4, and the P values are displayed in Table 3.

HLA Allele versus Monosomy 3

Since we had shown previously that an inflammatory phenotype is associated with loss of one chromosome 3 in uveal melanoma tissue, we performed a comparison between the presence of HLA alleles and monosomy 3 in these 45 cases. No significant associations were found between any of the HLA alleles and monosomy 3 (supplementary data, Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11–8901/-/ DCSupplemental).

Discussion

Certain HLA polymorphisms predispose to immunological and neoplastic diseases. The frequencies of HLA alleles found in our study group correlated well with known data of HLA frequencies in the Caucasian population.[40,42–44] We analyzed the most common HLA alleles, as well as HLA alleles that were associated previously with bad prognosis in uveal melanoma, and some alleles that have a known association with ocular inflammation. Individuals with local autoimmune disease might be protected against the development of melanoma due to local immunosurveillance. However, the alleles HLA-A29 and HLA-B40 were very infrequent, and HLA-B27 was absent in the 45 patients studied. Patients positive for HLA-DR4 (a gene with an association with VKH) had a trend toward a lower HLA-DR expression on their tumor cells, while the presence of the HLA-DR6 allele was associated with a higher HLA-DR expression on uveal melanoma tumor cells. With regard to macrophage infiltration, only the presence of the HLA-A2 allele correlated with less macrophage infiltration. After correction for the number of analyses, statistical significance was no longer present. Our study was performed to determine a possible relationship between HLA genotype and the level of HLA expression, and the amount of macrophage infiltration or loss of chromosome 3 in uveal melanoma. To study these relationships, we analyzed common alleles, as the results otherwise most likely would be insignificant due to necessary corrections for the number of analyses. Focusing on a

Table 3. HLA allele versus HLA expression and macrophage infiltration

			HLA-A expression (% HCA2+)			HLA-B and C expression (% HC10+)			HLA-DR expression (% Tal.1B5+)				CD68+ cells (mm^2x10^4)			CD68+CD163+ (mm^2x10^4)		
		n=45										*n*=38						
HLA allele		*n*	**Mean**	***P***	***P****	**Mean**	***P***	***P****	**Mean**	***P***	***P****	*n*	**Mean**	***P***	***P****	**Mean**	***P***	***P****
A1	**neg**	31	40			35			22			27	12.9			10.3		
	pos	14	51 ↑	.23	1.00	44 ↑	.57	1.00	18 ↓	.82	1.00	11	12.7↓	.83	1.00	9.1 ↓	.55	1.00
A2	**neg**	21	43			45			23			19	15.4			12.3		
	pos	24	44 ↑	.96	1.00	31 ↓	.12	.95	19 ↓	.55	1.00	19	10.3↓	**.03**	.26	7.6 ↓	**.02**	.14
A3	**neg**	29	41			34			19			23	11.9			8.8		
	pos	16	49 ↑	.30	1.00	43 ↑	.24	1.00	24 ↑	.36	1.00	15	14.4↑	.24	1.00	11.7 ↑	.14	1.00
B44	**neg**	35	43			38			19			31	13.0			9.9		
	pos	10	45 ↑	.82	1.00	34 ↓	.73	1.00	26 ↑	.33	1.00	7	12.0↓	.99	1.00	10.2 ↑	.87	1.00
Bw4	**neg**	22	44			38			18			20	12.7			9.7		
	pos	23	43 ↓	1.0	1.00	37 ↓	.67	1.00	23 ↑	.68	1.00	18	13.1	.75	1.00	10.1 ↑	.93	1.00
Bw6	**neg**	7	42			36			31			5	10.1			8.6		
	pos	38	44 ↑	.99	1.00	38 ↑	.90	1.00	19 ↓	.55	1.00	33	13.3↑	.59	1.00	10.1 ↑	.62	1.00
DR4	**neg**	32	44			40			22			29	13.8			10.5		
	pos	13	43 ↓	.88	1.00	32 ↓	.46	1.00	17 ↓	.06	.46	9	9.9↓	.19	1.00	8.0 ↓	.20	1.00
DR6	**neg**	31	47			37			16			26	13.2			9.8		
	pos	14	36 ↓	.32	1.00	39 ↑	.70	1.00	31 ↑	**.04**	.33	12	12.2↓	.85	1.00	10.3 ↑	.68	1.00

HLA expression and amount of macrophage infiltration per HLA allele. All tests are performed with the Mann-Withney-U (Wilcoxon rank) test.

Arrows represent graphically whether there was a higher or lower expression or a higher or lower amount of cells, per positive HLA allele.

* P-value corrected for number of tests (*n*=8).

limited number of frequently-occurring alleles would reveal population-relevant correlations.

The relationship that we observed between the HLA-DR4 and the HLA-DR6 allele, and level of HLA-DR expression could suggest that certain HLA genotypes influence the expression of HLA on tumor cells. Since we know that a high expression of HLA classes I and II are prognostic factors for metastasis in uveal melanoma,[13,15] this would be expected. However, the tendency to a lower expression associated with HLA-DR4 is surprising, as this gene is associated with inflammation through its link with VKH.

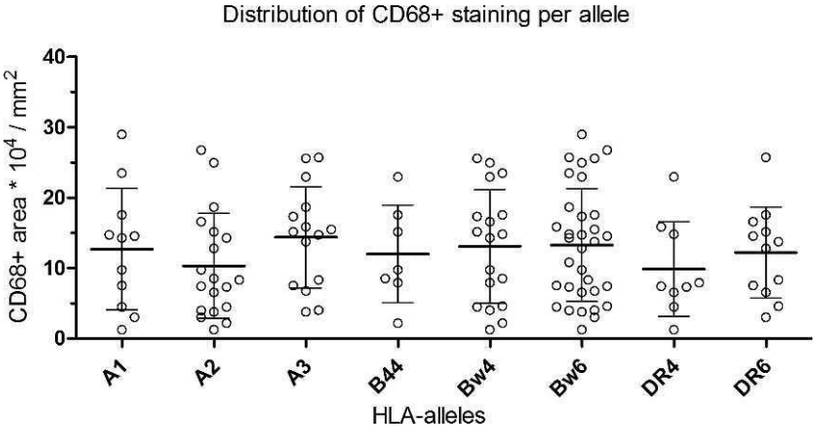

Figure 3. Distribution of CD68-positive staining for the most common HLA alleles. Error bars represent the SD of the mean.

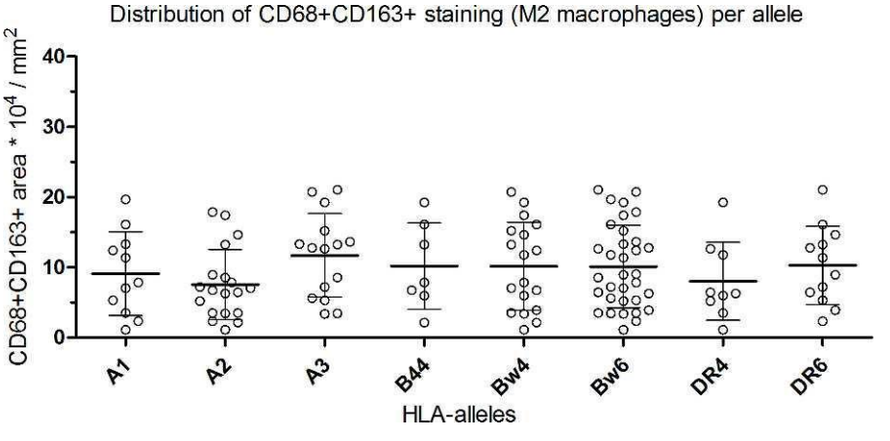

Figure 4. Distribution of CD68 and CD163 positive staining, representing the M2 macrophages, for the most common HLA alleles. Error bars represent the SD of the mean.

It is known that different mechanisms lead to HLA down regulation, which may be allele specific or haplotype specific.[45] While mutations in the β2-microglobulin (β2M) gene can be responsible for lack of HLA class I expression in colon carcinoma and cutaneous malignant melanoma,[46–48] in uveal melanoma no complete loss of β2M has been found, although a significantly decreased expression of

β2M has been described[15] and is associated with a favorable outcome.[49] Loss of chromosome material due to defective chromosome segregation or mitotic recombination may be another mechanism for loss of HLA expression. However, a study on the presence of loss of heterozygosity (LOH) in the area on chromosome 6 that codes for the HLA class I genes did not find a correlation of LOH with HLA-A or -B expression.[50] Up regulation of HLA classes I and II expression also is mediated by cytokines, such as interferon-alpha and –gamma,[45,51] which are released in the presence of an inflammatory infiltrate. Inflammation often is found in uveal melanoma and is associated with a worse prognosis.[18,52]

The observed correlation between HLA-A2 and fewer infiltrating macrophages in uveal melanoma is difficult to interpret. Less macrophage infiltration is associated with a better prognosis.[29–31] Thus, the association between being positive for the HLA-A2 allele and fewer infiltrating macrophages pleads for a protective function of the HLA-A2 allele in uveal melanoma. However, studies have shown that a direct association for HLA-A2 with survival in uveal melanoma does not exist.[26,27] This does not exclude any influence of HLA-A2 on macrophage infiltration and, to prove this, studies with a larger patient population should be undertaken.

No cases were positive for HLA-B27, which is known for its association with several autoimmune diseases, that is ankylosing spondylitis and anterior uveitis. This most likely is due to the relatively small study group, as a previous association study showed that 7% of 235 uveal melanoma patients were positive for HLA-B27.[27] We also looked at HLA-A29 because of its association with VKH, and at the HLA-B alleles B44 and B40 (now B60 and B61), as earlier studies showed an increased risk for metastases with these alelles,[26,27] although no association with an increased susceptibility to uveal melanoma was seen.[53] However, most of these alleles occurred with a frequency that was too low for a useful comparison. Where possible, we did analyze relations with these alleles, and did not find a correlation with HLA expression and macrophage infiltration or chromosome 3 status.

Understanding the pathological mechanism of HLA expression and macrophage infiltration in uveal melanoma is important, since both are related to prognosis. Our study shows that with the possible exception of an association between HLA-A2 and fewer infiltrating macrophages, and between HLA-DR6 and increased HLA-DR expression, HLA genotype does not determine the amount of HLA expression and macrophage infiltration or chromosome 3 status in uveal melanoma. It is important to look at these findings in the light of multiple testing, as these associations were observed before correction of the P value because of the number of tests performed. A second study with larger patient population, analyzing HLA genotypes with HLA expression and macrophage infiltration, will be essential to determine whether the associations we observed were valid.

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CHAPTER 6

Upregulation of HLA Expression in Primary Uveal Melanoma by Infiltrating Leukocytes

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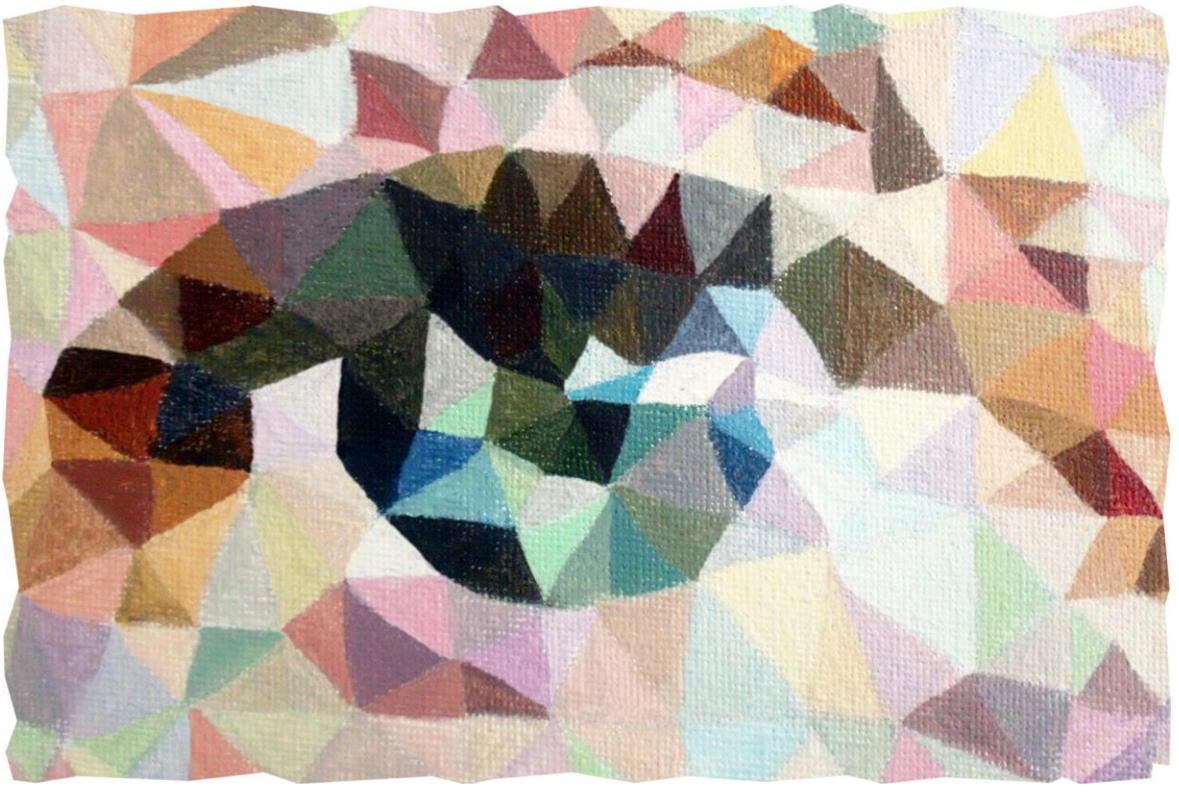


Abstract

Introduction: Uveal melanoma (UM) with an inflammatory phenotype, characterized by infiltrating leukocytes and increased human leukocyte antigen (HLA) expression, carry an increased risk of death due to metastases. These tumors should be ideal for T-cell based therapies, yet it is not clear why prognostically-infaust tumors have a high HLA expression. We set out to determine whether the level of HLA molecules in UM is associated with other genetic factors, HLA transcriptional regulators, or microenvironmental factors.

Methods: 28 enucleated UM were used to study HLA class I and II expression, and several regulators of HLA by immunohistochemistry, PCR microarray, qPCR and chromosome SNP-array. Fresh tumor samples of eight primary UM and four metastases were compared to their corresponding xenograft in SCID mice, using a PCR microarray and SNP array.

Results: Increased expression levels of HLA class I and II showed no dosage effect of chromosome 6p, but, as expected, were associated with monosomy of chromosome 3. Increased HLA class I and II protein levels were positively associated with their gene expression and with raised levels of the peptide-loading gene *TAP1*, and HLA transcriptional regulators *IRF1*, *IRF8*, *CIITA*, and *NLRC5*, revealing a higher transcriptional activity in prognostically-bad tumors. Implantation of fresh human tumor samples into SCID mice led to a loss of infiltrating leukocytes, and to a decreased expression of HLA class I and II genes, and their regulators.

Conclusion: Our data provides evidence for a proper functioning HLA regulatory system in UM, offering a target for T-cell based therapies.

Introduction

Uveal melanoma (UM) is the most common primary intraocular tumor in adults with an incidence of 5.1 per million.[1] Metastases develop in up to 50% of patients,[2] and there is still no effective cure for metastatic disease. Once the tumor has metastasized, the median patient survival is approximately one year, and survival has remained largely unaltered during the last 40 years.[1] A patient's individual prognosis is easily predictable, as during the last three decades several reliable prognostic markers have been found for UM and tests predicting patient survival have been developed. These include gene-expression profiling (GEP),[3] and chromosome testing: patients that have lost one copy of chromosome 3 (monosomy 3) are at high risk to die from metastatic disease.[4,5] Other chromosomal aberrations which modulate the risk of metastasis are 6p gain[6,7] and 8q gain.[8-10] Gain of 6p is only rarely observed in the same tumor as monosomy 3, and is associated with a favorable prognosis, while gain of 8q is often associated with monosomy of chromosome 3 and associated with a bad prognosis.[6,7]

Inflammation is another prognostic marker for the development of metastatic disease,[4,11] and is associated with monosomy 3.[4,12] Inflammation has been established as one of the hallmarks of cancer,[13] and despite the fact that the eye is an immune-privileged site, inflammation can be present within the intraocular tumor. The inflammatory phenotype seen in UM is characterized by an increased number of tumor-infiltrating lymphocytes and macrophages, as well as an increased expression on UM cells of Human Leukocyte Antigens (HLA) class I and II.[4] Several studies already showed that, in contrast with many other malignancies, a high expression of the HLA class I and II antigens is associated with a poor prognosis.[15-16] The sensitivity of UM to natural killer (NK)-cell mediated lysis was shown to be inversely correlated to this HLA protein expression:[15,17] UM cells with a high HLA class I expression gave rise to higher numbers of metastases in mice, which suggests that the tumor cells are able to escape from NK-cell mediated lysis. As especially UM with a high HLA expression give rise to metastases in patients, this possible escape mechanism may also be present in humans.[18]

The genes encoding both the HLA class I and II antigens are located on chromosome 6p. This faces us with an intriguing paradox because in general, chromosomal gain tends to lead to an increased gene expression in tumors.[19,20] While in UM, 6p gain is associated with a good prognosis, an increase in HLA expression of both classes is associated with a poor prognosis. This made us wonder what exactly regulates HLA expression levels in UM: chromosomal dose effects (gain or loss of chromosome 6), an intrinsic genetic regulation, or external influences. We know that occasionally specific HLA alleles are not expressed.[21] Therefore we looked in a previous study at the association between HLA expression and LOH (Loss of Heterozygosity) of chromosome 6.[22] However, no relation between LOH and lack of expression was observed at the time, suggesting that a low HLA expression was not caused by loss of chromosome 6 material. Furthermore, several studies have

shown that the more malignant UM are not characterized by a downregulation, but by an upregulation of HLA class I and II antigens.[14-16]

While expression at the cell surface depends on a properly-functioning peptide-loading system, regulation at the gene level depends on a set of different genes, which includes *NLRC5* and *CIITA*. *NLRC5* plays a crucial role in the transcriptional regulation of HLA class I genes,[23] and *CIITA* in the transcriptional regulation of the *HLA class II genes,*[24] while it is also involved in *HLA class I* transcriptional activation.[25] The promoters *NLRC5* and *CIITA* are on their turn under the influence of, amongst others, the interferon-regulatory factor 1 (IRF1).[26] Furthermore, Holling et al., using UM cell lines, reported that HLA class II could be induced in half of their UM cell lines, and showed that the lack of HLA class II expression in one particular cell line was caused by epigenetic silencing of the gene encoding CIITA. Silencing of *CIITA* was mediated through EZH2 (Enhancer of Zeste Homologue 2, a Polycomb Repressive Complex 2 subunit; chr7q), which triple methylates lysine 27 in histone H3.[27] That not only transcriptional regulators influence HLA class I and II expression, but also external influences, was also shown using UM cell lines where interferon-gamma (IFNG) stimulation led to increased levels of HLA class I and II.[27,28] Additionally, downregulation could be induced by tumor growth factor beta (TGFB).[17,28] However, all of these studies regarding the regulation of HLA expression were performed on a limited number of available cell lines. As IFNG induces upregulation of HLA molecules in cell lines *in vitro*, IFNG produced by tumor-infiltrating leukocytes may have a similar effect *in vivo* in high-risk UM. HLA expression is of great importance for T-cell based therapies, because without HLA-molecules, T-cells cannot react to and subsequently destroy their target cells.[29] Therefore it is important to determine whether HLA expression in UM cells functions properly, and how it is regulated.

We here investigate whether chromosomal dose effects or specific known regulators influence HLA gene or protein expression in UM, this time studying primary enucleated tumors and not cell lines. We analyze the relationship between HLA class I and II RNA and protein levels, and genes involved in the regulation of (HLA) transcription, genes of the peptide-loading system, such as the TAP molecules, and the influence of the absence or presence of one chromosome 3 or 6p. Additionally, we assess the association and influence of the microenvironment on HLA gene expression by comparing expression levels in human primary or metastatic UM with their corresponding xenografts placed in mice, which lack tumor-infiltrating leukocytes.

Methods

Study Population

Tumor tissue was obtained from 28 eyes that underwent primary enucleation for UM between 1999 and 2004 at the LUMC in Leiden, the Netherlands. Patient records and survival were updated from the patient's charts and through the Dutch National Registry; the last update was in November 2013.

The current study population has previously been described,[30] but only cases with mate rial suitable for gene-analysis were included; each tumor sample was processed for conven tional histopathological evaluation, including cell type assessment according to the modified Callender classification. The mean age at enucleation of the 28 patients was 62 years (median 68, range 28–84 years) and median follow up was 72 months (median 76, range 14–145 months). At the time of the study, 13 patients were alive, 13 had died due to UM metastases, and in two cases, the cause of death was unknown. The mean time to metastasis was 37 months (median 30, range 14–96 months).

SNP and gene expression

A genome wide micro-array analysis on single-nucleotide polymorphisms (SNPs) was performed with the Affymetrix 250K Nsp array (Affymetrix, Santa Clara, CA, US) to acquire a highly detailed image of the chromosome copy numbers. The average number of copies of all genes lying on the short (p) or long arm (q) of chromosome was used to determine gain or loss of that arm. For chromosome 6p we focused on gain or loss of the HLA region on that arm.

Gene-expression profiling was performed with the Illumina HT12 v4 array (Illumina, Inc., San Diego, CA, US) for the HLA genes (*HLA-A*, *-B*, *beta-2-microglobulin*, *HLA-DR [HLA- DRA*, *HLA-DRB1*, *HLA-DRB3*, *HLA-DRB6]* and *HLA-DQ [HLA-DQA1*, *HLA-DQB1]*), the genes encoding HLA transcriptional regulators (*NLRC5,*[25,26,31] *CIITA,*[25,26,31] *IRF8*, *IRF1*, *IRF2*and genes of the peptide-loading machinery (*TAP1*, *TAP2*, *PDIA3*, *Tapasin*, *Calreticulin*). Beta-2-microglobulin(B2M) is a component of the HLA class I complex at the cell surface, and should theoretically be upregulated when there are more HLA-molecules on the cell surface. However, the B2M gene is located on a different chromosome (15q21). HLA-C was not analyzed, as it has a tenfold-lower expression at the protein level than HLA-A and –B.[32,33] As the peptide-loading mac hinery is needed to get a functional HLA class I molecule expressed at the cell surface, we looked at several of these genes. In cases where Illumina provided multiple probes for the same gene, the mean value of these probes was used for further analysis. One of four probes for HLA-A (ILMN_2165753) was omitted because of discordancy with the values of the other three HLA-A probes.

The Illumina array data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus[34] and are accessible through GEO Series accession number GSE84976 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE84976).

Validation

Quantitative polymerase chain reaction (qPCR) was performed, as described previously,[35] in duplicate on the selected genes for HLA and HLA-regulation activators, as a validation of the results found with gene-expression profiling.

In short, RNA was extracted from the fresh frozen primary tumors using an RNeasy Mini Kit (Qiagen). Complementary DNA (cDNA) was synthesized with the iScript cDNA synthesis kit (Bio-

Rad). Beacon Designer (Biosoft) was used for primer design. Primers for *ACTB (β- actin;* OMIM 102630), *GAPDH* (OMIM 138400), *RPL13* (OMIM 113703), and *RSP11* (OMIM 180471) were included for selecting suitable reference genes. Gene expression was calculated by normalizing the C-value of each marker to the reference genes. Reference genes were selected based on their stable expression in the tissue, which was measured with the geNorm Software.[36]

In the material studied, *RPL13* and *RPS11* were stably expressed, and the genes of interest were corrected for the geometric mean of these two reference genes as described previously.[37] For a overview of the primers used, see the supplements (S1 Table).

Immunohistochemistry

Immunohistochemical staining for HLA class I (HLA-A, -B) and II (HLA-DR) was previously performed as described.[4] In short, immunohistochemical staining was carried out using the mouse monoclonal antibodies HCA2 (Produced by the Netherlands Cancer Institute, Amsterdam, the Netherlands) exclusively staining HLA-A heavy chains, HC10 (Produced by The Netherlands Cancer Institute, Amsterdam, the Netherlands) which binds to HLA-B and -C heavy chains,[38,39] and Tal.1B5 (DakoCytomation, Glostrup, Denmark) reacting with HLA-DR alpha chains. The number of tumor cells positive for each marker was counted at 100X magnification and expressed as percentage of the total number of tumor cells (S2 Table).

Immunofluorescence staining for infiltrating macrophages was performed and described previously, with antibodies against CD68 (clone 514H12; Abcam, Cambridge, UK) and CD163 (clone 10D6; Novocastra, Newcastle-upon-Tyne, UK), and expressed as pixels per millimeter squared.[40] T cells were identified and scored previously using an anti-CD3 antibody (ab828; Abcam), and expressed as numbers of cells per millimeter squared.[12]

Xenografts

Fresh intraocular or metastatic human tumor samples were acquired with informed consent at the Institut Curie (Paris, France), and immediately transplanted into the interscapular fat pad of two to four non-preirradiated immunodeficient female SCID mice, and considered to be stable xenografts for characterization after three consecutive mouse-to-mouse passages.[41]

Total RNA was isolated from frozen material of the fresh intraocular and metastatic human tumor samples, and from frozen material of the corresponding xenografts, and subsequently analyzed with GeneChip Human Exon 1.0 ST microarrays (Affymetrix).[42]

Of these tumors, eight of primary and four of metastatic origin were included in our study and expression on the microarray was analyzed for *HLA-A*, *HLA-B*, *HLA-DR (HLA-DRA)*, and *HLA-DQ (HLA-DQA1*, *HLA-DQA2)*, and markers of infiltrating T cells: *CD3 (CD3D*, *CD3E)*, *CD4)*, and *CD8 (CD8A*, *CD8B)*, and macrophages: *CD68*, and *CD163*.

Statistics

Data analysis of the material obtained at the LUMC in Leiden was performed with the statistical programming language R version 3.0.1 (R: A Language and Environment for Statistical Computing, R Core Team, R foundation for Statistical Computing, Vienna, Austria, 2014, http://www.R-project.org), supplemented with specialized packages for SNP and RNA analysis. The main package used for SNP analysis was aroma.affymetrix,[43-45] supported by 'DNA-copy' (Venkatraman E. Seshan and Adam Olshen, DNAcopy: DNA copy number data analysis. R package version 1.34.0), 'sfit' (Henrik Bengtsson and Pratyaksha Wirapati (2013), sfit: Multidimensional simplex fitting. R package version 0.3.0/r185, http://R-Forge.R-project.org/ projects/matrixstats/), and 'R.utils' (Henrik Bengtsson (2014), R.utils: Various programming utilities, R package version 1.29.8, http://CRAN.R-project.org/package=R.utils). Data of eighty-four healthy controls served as reference set, obtained with the same Affymetrix 250K Nsp chip (Affymetrix, Santa Clara, CA, USA) by the Department of Human Genetics at our center. The 'Aroma.Affymetrix' package made it possible to use these SNP microarrays to determine copy number values.[43-45] The packages used for RNA microarray analysis were 'limma' version 3.16.8 and the specific packages for Illumina microarrays: 'lumi' version 2.12.0, 'annotate' (R. Gentleman, annotate: Annotation for microarrays, R package version 1.38.0), and 'illuminaHumanv4.db' (Mark Dunning, Andy Lynch and Matthew Eldridge, illuminaHumanv4.db: Illumina HumanHT12v4 annotation data (chip illuminaHumanv4), R package version 1.18.0).

Data of the xenografts and corresponding original human tumors were analyzed at the Institut Curie (Paris, France) with R software (version 2.12) at gene level using custom Brain array Chip Description Files (CDF) based on Entrez Gene database (version 13) as described in detail by Laurent et al.[42] For this study, a paired sub-analysis was made at the Institut Curie with regard to the genes of our interest.

All other statistical analysis was performed with SPSS 20.0.1 (IBM SPSS Statistics, IBM Corporation, Armonk, NY, US). Cumulative survival was calculated with Kaplan-Meier and its significance analyzed with the log rank test. Hazards ratios (HR) were measured with Univariate Cox regression models. Associations for clinical categorical variables with gene expression were determined using the Mann-Whitney U test (Wilcoxon rank sum test) for non-parametric analysis. Statistical correlation between two continuous variables was calculated with the Spearman's rank test. For survival analysis with Kaplan-Meier, the linear variables were dichotomized at 50% cut off, unless mentioned otherwise. Statistical significance was assumed for a p-value <0.05.

Study approval

The collection of material and the research performed with it for this study has been agreed upon by the Medical Ethics Committee of the LUMC (Leiden University Medical Center, Leiden, the Netherlands). The research protocol adhered to Dutch law and the current version of the tenets of the Declaration of Helsinki (World Medical Association of Declaration 1964; ethical principles for medical research involving human subjects). Patients were informed about the use of their

enucleated eyes for research, and signed an informed consent prior to the enucleation. The performance of the xenograft study was in accordance with the French Ehtics Committee (Ministère de l'Alimentation, de l'Agriculture et de la Pêche, Direction de la Santé et de la Protection Animale, Paris, France). Both studies were thus specifically approved by the Ethics Committee of the respective centers, the first one being the Medical Ethics Committee of the LUMC (Leiden University Medical Center, Leiden, the Netherlands) and the second one being the just mentioned French Ethics Committee (Ministère de l'Alimentation, de l'Agriculture et de la Pêche, Direction de la Santé et de la Protection Animale, Paris, France).

Results

HLA gene expression and HLA immunohistochemistry

We wondered whether differences that were noticed in *HLA* gene-expression assays correlated with previous immunohistochemical staining results on tumor cells and observed that the gene-expression of *HLA-A* and *-B* was significantly correlated with the percentage of tumor cells being, respectively, positive for HCA2 (HLA-A) and HC10 (HLA-B/C) (Table 1). *B2M* gene-expression correlated positively with HCA2 and HC10 staining too, although not significant for HCA2. Gene-expression of *HLA-DR* failed to correlate with monocolonal antibody Tal.1B5. Gene-expression levels of the HLA molecules in the Illumina test correlated with death due to metastases (S3 Table). When comparing clinical and histopathological parameters, gene expression of *HLA-A*, *HLA-B*, *B2M*, and *HLA-DR* was positively associated with age at enucleation and with the largest basal diameter of the tumor, a well-known adverse parameter.

The genes that code for the HLA class I and II antigens are located on chromosome 6p, which has prognostic importance in UM. In order to determine whether the level of HLA class I and II antigens was influenced by a gene-dosage effect, we determined the presence of chromosome 6 by SNP array, and compared HLA and B2M gene expression levels with chromo- some dosage. Additionally, we examined the gene-dose effect of chromosome 3, as a previous study from our laboratory had indicated an association between loss of chromosome 3 and increased HLA expression levels on primary UM(8). An overview of the clinical, chromosomal, gene-expression, and histological data is provided in S4 Table.

Aberrations of chromosome 3 and 6p

Aberrations of chromosome 3 and 6p were analyzed with a SNP micro-array in 28 UMs. Monosomy 3 was present in fourteen (50%) cases and associated with death due to metastases (Kaplan-Meier, $p < 0.001$).

Gain of the HLA region of chromosome 6p was present in eight cases (29%), and associated with good survival (Kaplan-Meier: $p = 0.049$). Gain of 6p co-occurred with monosomy 3 in only one case.

Table 1. Correlation of gene-expression with immunohistochemistry for HLA

	mAb HCA2 (HLA-A)		mAb HC10 (HLA-B/C)		mAb Tal.1B5 (HLA-DR)	
Gene-expression (Illumina)	*r*	*p*	*r*	*p*	*r*	*p*
HLA-A	.434	0.02	.545	0.003	.161	0.41
HLA-B	.488	0.01	.609	0.001	.286	0.14
B2M	.346	0.07	.508	0.006	.279	0.15
HLA-DR	.242	0.21	.332	0.08	.225	0.25
HLA-DQ	.301	0.12	.400	0.04	.172	0.38

Correlation of HLA gene-expression, as determined with the Illumina array, with the results of HLA immunohistochemical staining in 28 cases of UM. *r* = two-tailed Spearman correlation coefficient. *p* = p-value.

To determine a possible dose effect of chromosome 6 on HLA expression levels in UM, we investigated whether there was an association of chromosome 6p dose and gene or protein expression of HLA. Monosomy of chromosome 3 was associated with an increased gene expression of *HLA class I* and *B2M* but not of *HLA class II*. Gain of 6p occurred almost exclusively in tumors without monosomy 3, and when looking at all tumors, an association was observed between 6p gain and less *HLA-B* expression (p = 0.049). Expression of HLA class I and II as determined by immunohistochemistry was not associated with 6p gain (HLA-A p = 0.12; HLA-B/C p = 0.26; HLA-DR p = 0.24).

However, the effect of the loss of one copy of chromosome 3 may have overshadowed that of chromosome 6p. Therefore we separated monosomy 3 and disomy 3 tumors into those with or without gain of 6p. As the group with monosomy 3 with gain of 6p consisted of only one tumor, we did not use this case for analysis. As expected, monosomy 3 tumors showed a higher expression of HLA class I than disomy 3 tumors, at the gene-expression as well as the protein- expression level (Fig 1A and 1B). When looking at the effect of 6p gain in disomy 3 tumors, no significant differences in gene and protein expression were observed between tumors with a normal 6p (n = 7) or a gain of 6p (n = 7). Similarly, HLA class II gene and protein expression was not different as well. We can conclude that there is no clear dose effect of chromosome 6p on HLA class I and II expression.

Peptide-loading genes and HLA transcriptional regulators

As chromosomal gain of 6p had no influence on the expression levels of *HLA* genes, we questioned whether HLA expression was influenced by the peptide-loading machinery or any of the known HLA regulators.

We compared expression levels of the *HLA class I* subtypes with expression of the genes encoding the main molecules of the peptide-loading machinery, eg. with *TAP1*, *TAP2*, *tapasin* and

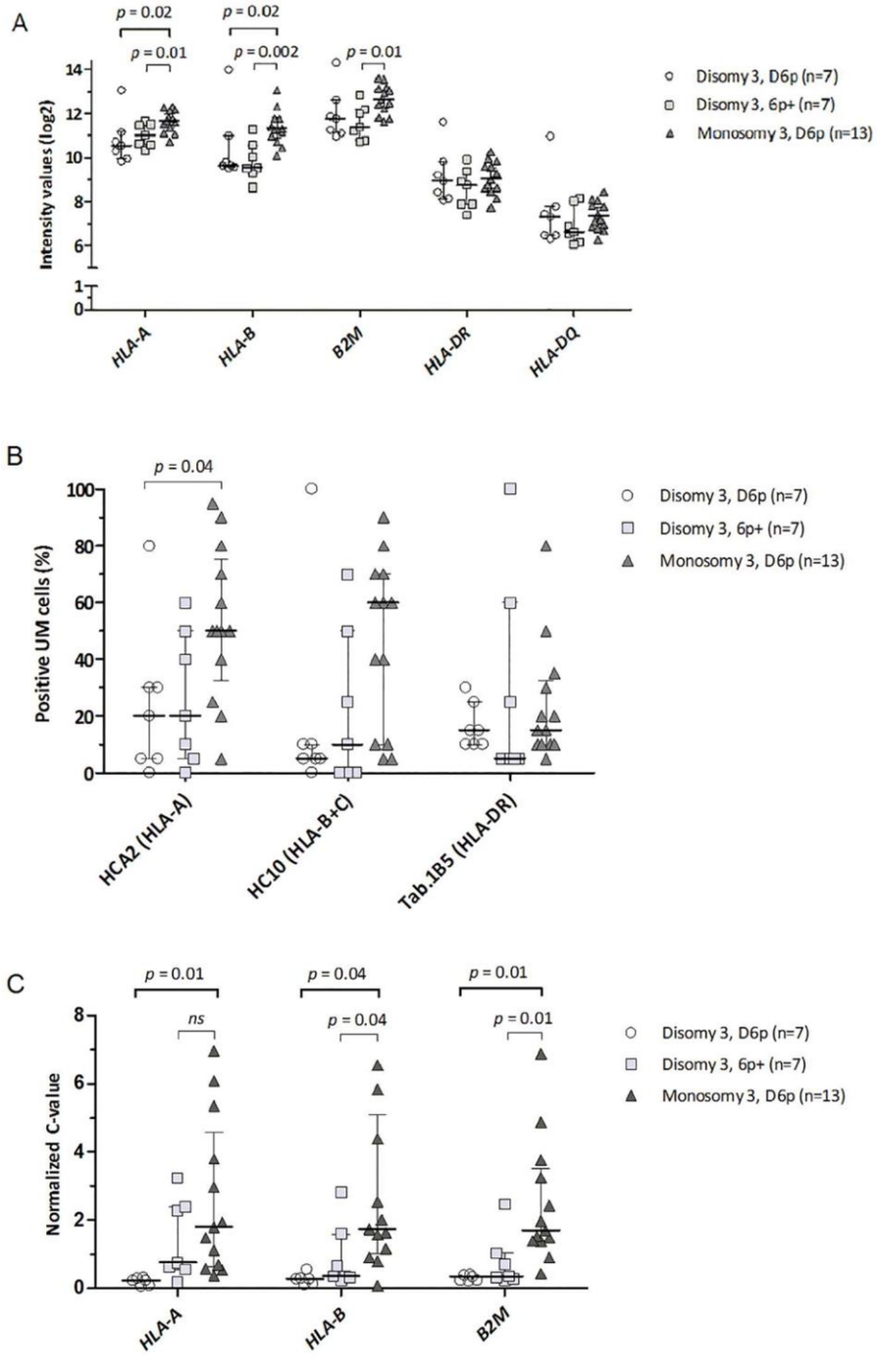


Figure 1. Chromosomal aberrations and HLA expression. Comparison between chromosomal aberrations and the expression of HLA class I and II antigens in a set of 27 primary UM. Tumors are divided according to their chromosome 3 and 6 status (disomy or monosomy of chromosome 3, and disomy of chromosome 6 or gain of 6p). HLA gene expression was determined using an Illumina microarray (A) and protein expression by immunohistochemistry (B) in UM. Additionally, HLA gene expression was determined using qPCR, which served to validate the Illumina findings (C). Four data points of the qPCR that are outside the axis limits (> 11 and < 24) are not shown (HLA-A, D3D6p: 17; HLA-B, D3D6p: 24, and M3D6p: 12; B2M, D3D6p: 13). Only significant p-values are shown, all other comparisons between the groups were not significant (p-values not shown). Error-bars represent the interquartile range. Results were obtained using the Mann-Whitney U tests.

calreticulin. *TAP1*-gene expression correlated positively with the gene-expression levels of *HLA class-I* and *B2M*, while *TAP2* in general showed a similar correlation, although this was not significant for all comparisons (Table 2). The other antigen-presenting machinery molecules were not co-regulated with HLA antigen expression.

We subsequently focused on the influence of known regulators of HLA gene expression. NLRC5 is a regulator for *HLA class I* and not for *HLA class II* genes, while CIITA is essential for the transcriptional regulation of HLA class II genes. In contrast to NLRC5, CIITA also plays an ancillary function in the transcriptional regulation of HLA class I genes. Other known HLA regulators, influencing both class I and II, are IRF1, IRF2, and IRF8. When we compared expression of these HLA transcriptional regulators with HLA expression in tissue of human uveal melanoma, we observed that the gene-expression levels of the HLA transcriptional activators *IRF1* and *IRF8* correlated positively with *HLA class I* and *B2M*, *NLRC5* with *HLA-B* and *B2M* expression, and *CIITA* with *HLA-B* as well as with *B2M* (Table 2). Additionally, the gene-expression levels of *IRF1*, *IRF8*, *NLRC5* and *CIITA* correlated positively with the expression levels of both *HLA class II* subtypes, i.e. *HLA-DR* and *HLA-DQ*. IRF1 and IRF8 exert their function on HLA class II genes via their involvement in CIITA transcription.

Table 2. Correlation between *HLA*- gene expression and the expression of the genes encoding the HLA transcriptional regulators and peptide-loading machinery

	HLA-A		*HLA-B*		*B2M*	
Peptide loading machinery	*r*	*p*	*r*	*p*	*r*	*p*
TAP1	.859	<0.001	.809	<0.001	.814	<0.001
TAP2	.367	0.06	.350	0.07	.465	0.01
PDIA3	.204	0.30	.184	0.35	.159	0.42
Tapasin	.171	0.38	.135	0.50	-.038	0.85
Calreticulin	-.036	0.86	.031	0.88	-.218	0.27

	HLA-A		*HLA-B*		*B2M*		*HLA-DR*		*HLA-DQ*	
HLA transcriptional regulators	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
CIITA	.276	0.16	.407	0.03	.408	0.03	.415	0.03	.465	0.01
NLRC5	.280	0.15	.470	0.01	.389	0.04	.557	0.002	.492	0.01
IRF1	.758	<0.001	.854	<0.001	.853	<0.001	.828	<0.001	.852	<0.001
IRF2	.137	0.49	.096	0.63	.179	0.36	.032	0.87	-.085	0.67
IRF8	.578	0.001	.679	<0.001	.689	<0.001	.937	<0.001	.916	<0.001

Correlations based on Illumina array data. *r* = two-tailed Spearman correlation coefficient. *p* = p-value.

Only an increased gene-expression level of *IRF1* was associated with monosomy 3, expression levels of *IRF2*, *IRF8*, *NLRC5*, and *CIITA* were not associated (S1 Fig).

A high expression of *TAP1* and of *IRF1* was associated with death due to metastases (*TAP1*: Univariate Cox regression: HR = 1.6, $p = 0.004$, and Kaplan-Meier: $p < 0.001$; *IRF1*: Univariate Cox regression: HR = 1.4, $p = 0.06$, and Kaplan-Meier: $p = 0.007$). *NLRC5*, *CIITA*, *IRF2*, and *IRF8* displayed no association with survival.

Validation

Quantitative polymerase chain reaction (qPCR) on the same 28 UMs was performed to validate our findings based on the gene-expression values obtained with Illumina HT12 v4 array for HLA class 1 genes (Fig 1C). Unfortunately, a suitable primer pair for qPCR could not be developed for *IRF8*. qPCR confirmed most of the correlations between the *HLA* genes and *TAP1*, *CIITA*, *IRF1*, and *NLRC5* (Spearman correlations, S5 Table). Using qPCR data, *TAP2* was significantly correlated with the *HLA* genes.

Tumor-infiltrating immune cells

Expression of several HLA class 1 and 2 molecules, regulators of the antigen-presenting machinery, as well as several of the transcriptional regulators, are thus co-regulated in human UM samples, and the presence of most of these, showed an association with loss of one chromosome 3. We noticed that all of these molecules are part of the interferon-regulated pathway. As infiltrating cells are considered important sources of interferon and infiltrating leukocytes are also an important part of the inflammatory phenotype of UM, we compared HLA expression with infiltrating leukocyte density, using the Illumina *HLA* expression data. We looked at the presence of macrophages and CD3+ T-cells, as markers of the presence of an immune infiltrate. We separated the tumors into two groups, with a low or high infiltration, and compared expression levels of *HLA-A*, *HLA-B*, *B2M*, *TAP1*, *IRF1*, *IRF8* and *NLRC5* between these two groups. Overall, a high infiltrate was associated with increased expression of the genes related to HLA class I (Fig 2A and 2B).

Xenografts

The clear association between the presence of infiltrating leukocytes and *HLA-A*, *HLA-B*, *TAP1*, as well as the HLA regulator genes suggests that the presence of leukocytes is associated with the level of expression of all HLA-associated molecules. However, it may be that a high HLA expression attracts leukocytes to the site of inflammation, or it may be that the HLA expression is upregulated by cytokine production from the leukocytes. These two options can be tested by comparing intraocular UM with xenografts from the same tumors in mice. When human tumors are placed as xenografts into SCID mice, they loose their infiltrating human leukocytes over time due to their limited timespan,[46] and any environmental effect caused by these leukocytes disappears. If the HLA expression is upregulated due to leukocyte-produced interferon, its expression should go down in a

xenograft. Twelve xenografts, consisting of eight primary tumors and four metastases of UM, were analyzed and their gene expression levels were compared with the original human tumor tissue. This allowed us to compare the effect of the absence of infiltrating human leukocytes on the expression of the genes of our interest (Fig 3). Indeed, a decrease in infiltrating T cells was observed (*Cd3E* $p < 0.001$, *Cd3D* $p < 0.001$, *Cd4* $p < 0.001$, *Cd8A* $p = 0.001$, Cd8b $p = 0.34$). Also, the markers for macrophages, *Cd68* ($p < 0.001$) and *Cd163* ($p < 0.001$), were decreased in xenografts. In parallel, the xenografts showed a decrease of the HLA genes investigated, as well as of most of the peptide-loading machinery genes and transcription regulation genes (S6 Table).

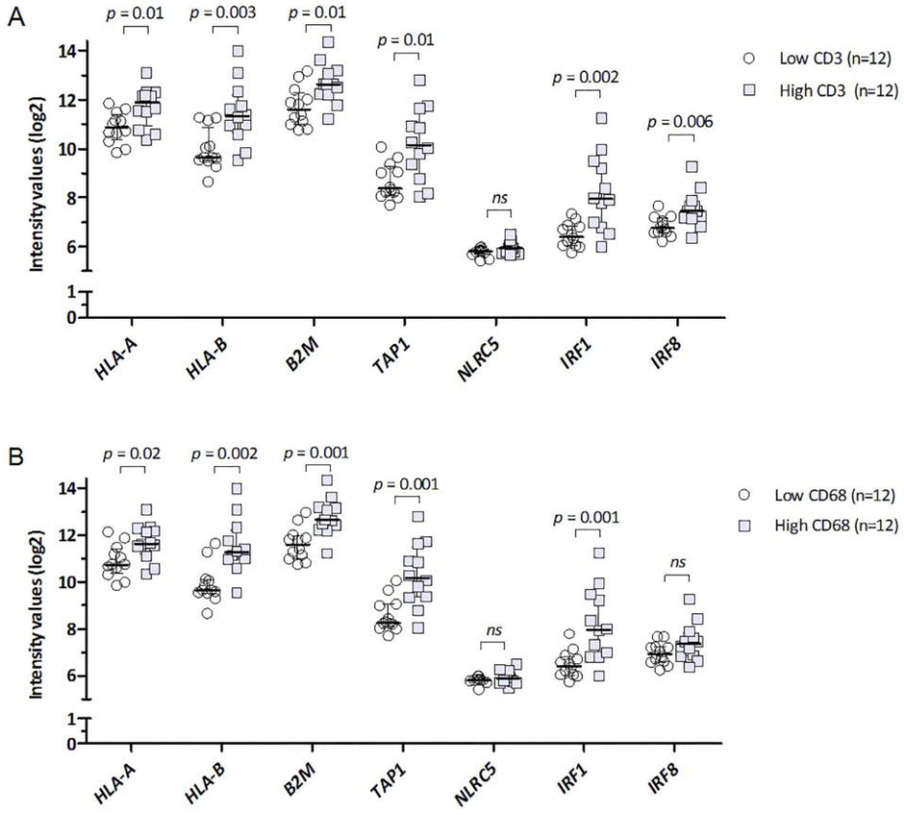


Figure 2. Tumor infiltrating immune cells. Association between a low or high density of tumor-infiltrating CD3+ (A), and CD68+ (B)cells and several HLA and HLA-related genes (Illumina array) in primary UM. CD3 (cells/mm^2) and CD68 (pixels x 10^3/mm^2) scores were dichotomized at the median.

Discussion

We found that the immune infiltrate was associated with an increased HLA-gene expression in UM. Immune infiltrate is associated with monosomy 3,[4,12] and loss of infiltrating human leukocytes from the xenografts in SCID mice resulted in a lower HLA class I and II gene expression. Many of the

studies on UM focus on markers predicting prognosis.[3,5,7,18,40] Predicting prognosis, important as it is, does not explain why and how these markers are related to an altered life expectancy. We first compared the relationship between the expression of HLA genes and aberrations of chromosome 3 and 6p, containing the HLA genes, on 28 extensively documented UM tumors.

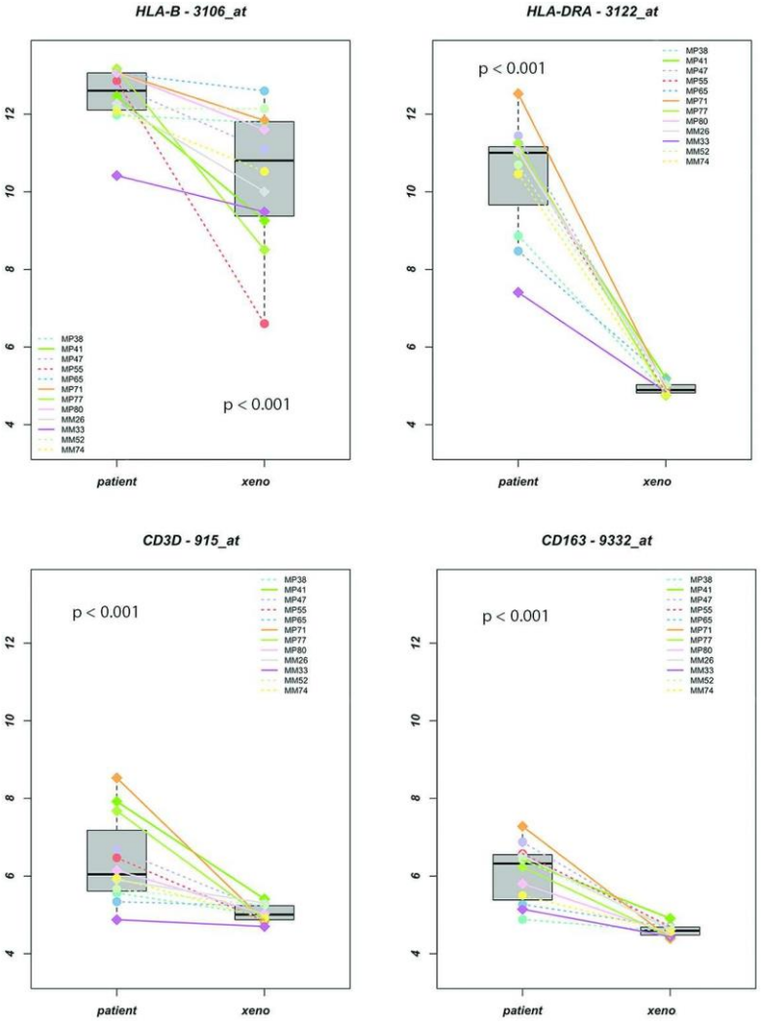


Figure 3. Effect of the absence of human leukocytes. Gene-expression (log 2 intensity values) of HLA-B, HLA-DRA, CD3D (as marker for T-cells), and CD163 (as marker for macrophages), of the original tumors (patient) compared to the xenografts (xeno). MP's are primary tumors; MM's are metasisis.

We observed that for the *HLA class I* and *II* genes, an increased number of copies of chromosome 6p led not to a higher HLA expression, showing that UM deviate from the general rule that gain of gene copy number leads to a higher expression of those genes (Fig 1). Following the line of HLA production from the presence of its complexes on the cell membrane downwards to the amount of RNA (gene-expression) and finally to the genes located in the nuclei, two patterns could be discerned: HLA class I gene and protein levels were not related to the dose effect of

chromosome 6p, yet they were clearly and inversely associated with the dose of chromosome 3. This not only affected the HLA class I and II antigens, but also transporter molecules, such as B2M located on a different chromosome than 6, as well as HLA expression regulators. In our earlier work on the same tumors,[4,12,22] we had used karyograms and FISH analysis on isolated nuclei for determining the presence of monosomy 3, while in the current study we used the more precise SNP array. We here show that loss of one chromosome 3 is associated with an increased leukocytic infiltrate, and increased expression of not only the HLA class I and II molecules, but also of molecules related to the peptide-loading machinery and transcriptional regulators.

The apparently intact HLA protein production tree in UM directed us to look outside the cell for factors influencing HLA expression. The HLA transcriptional regulators are under the influence of IFNG, a cytokine often produced by lymphocytes.[47] IFNG can activate Janus kinase 1 and 2 (JAK1 and JAK2), which causes activation of STAT1 (signal transducer and activator of transcription 1) and IRF1, leading to induction of CIITA.[26] With regard to HLA class I expression, NLRC5 has been identified as the key transcriptional regulator at this level in human cells of different origin (embryonic kidney cells, HeLa cells, and Jurkat T cells).[23] Our current findings have been obtained on a set of 28 primary UM, and show that not only TAP1 is being upregulated together with HLA, but that *HLA class I* is upregulated together with *TAP1*, *IRF1*, *IRF8*, *NLRC5* and *CIITA*, and *HLA class II* together with *CIITA* and *IRF1*, which agrees with the previous results obtained using cell lines.[27]

It was previously reported that EZH2 (also known as KMTase Enhancer of Zeste Homolog 2), a member of the polycomb repressive complex 2 (PRC2), is involved in blocking IFNG-induced upregulation of *CIITA* in a UM cell line.[27] Monosomy 3 in uveal melanoma is associated with loss of BAP1 (BRCA1 associated protein-1; chr3p),[8,48] which is connected to PRC2 (of which EZH2 is a member) through ASXL1(additional sex combs-like transcriptional regulator 1).[49,50] This could represent a possible connection between BAP1 and expression of HLA in uveal melanoma, as study in mice showed that loss of BAP1 leads to increased levels of EZH2 (and PRC2) with repressed expression of its targets,[51] including thus CIITA, leading ultimately to less HLA class II expression. However, this is not what we found in uveal melanoma, where BAP1 loss seemed to be associated with increased HLA expression.[52] The general picture that emerges from the Illumina array was validated with the qPCR (Fig 2). *HLA class I* and *HLA II* are upregulated in monosomy 3 tumors, and the upregulation of HLA class I genes is positively correlated to *IRF1*, *CIITA*, and *TAP1* (and *TAP2*), while *HLA class II* is upregulated together with *IRF1*, *CIITA* and *NLRC5*. *NLRC5* is located on the same chromosome as *CIITA* (chromosome 16). Since both of these transcriptional regulators belong to the NOD-like receptor family of proteins and share some regulatory elements in their promoters, it could therefore be a co-regulatory effect that explains this finding for NLRC5. Some UM are known to loose copies of Chromosome,[16,53] so likely here is no gene dose effect.

As we were not finding clues indicating that the regulation of HLA expression was internally deranged in these UM, we aimed to find the origin of the elevated HLA expression by factors outside the UM cells. The most well-known factor is IFNG, which has previously

been shown to stimulate HLA expression in UM cell lines,[28] IFNG is predominantly secreted by CD4+ T helper 1 cells (Th1), CD8+ cytotoxic T cells, and NK cells, although it is also to a lower degree produced by professional antigen-presenting cells (APCs), including macrophages, and B-cells. Our data confirmed that indeed a high leukocyte infiltration of CD68+ and CD3+ leukocytes, was associated with an increase in gene expression of *HLA*, *B2M*, *TAP1*, and *IRF1*. Some of these markers may be more highly expressed at the RNA level due to the presence of leukocytes in the RNA that was used for the array and qPCR. One may suggest that the PCR data identified HLA antigens on infiltrating cells, however all original studies were performed using immunohistochemistry on tumor sections, analyzing melanoma-cell specific HLA class I and II and B2M expression. We can thus conclude that we are really looking at the HLA expression of the tumor cells themselves.

Using SCID-mice (lacking T and B cells) into which freshly-obtained UM were grafted, we observed that xenografts obtained from tumors that originally showed a high *HLA class I and II* expression, showed a much lower *HLA class I and II* expression than their original counterpart (Fig 3). The original tumors (primary and metastatic) were usually monosomic for chromosome 3 and exhibited high gene-expression levels of *CD3*, *CD4*, *CD8* (T-cell markers), and *CD68* and *CD163* (macrophages markers), as well as a high HLA class I and II gene expression. The average expression of all of these genes decreased in the xenografts, although the tumors continued to show monosomy of chromosome 3. This means that the tumor-infiltrating lymphocytes (TILs), and especially the T cells as main producers of IFNG, are the ones triggering the increased HLA expression in UM. Jehs et al. observed that UM cell lines are not producing IFNG themselves, and that upon co-culture with IFNG-secreting T cells, the UM cells were responding by synthesizing chemokines that create a tumor-promoting environment with increased HLA expression and attraction of M2 type macrophages.[54] Indeed, the T cell-containing primary UMs as well as the xenografts contained high numbers of macrophages. Following xenografting, a decrease in gene-expression of *CD68* was observed.

Although the question remains how these T cells are exactly recruited to the tumor, and whether they do or do not recognize the peptides presented in the HLA molecules, all these data together indicate that the presence of an infiltrate is a bad prognostic parameter in uveal melanoma. Several studies have shown that UM contain Foxp3-positive regulator cells, which may inhibit effective CTL function.[12,55] An interesting option is to specifically deplete these regulator T cells. A recent study in a mouse model showed that attacking a T-reg specific kinase may help bring back CTLs, which subsequently kill the tumor cells.[56] As T cells may produce factors that bring in macrophages,[54] T-cell depletion may also reduce macrophages density, and this may subsequently influence the development of the primary tumor or metastases, as the presence of macrophages is related to angiogenesis and most of the macrophages in UM are of the pro-angiogenic M2 type.[40] This and other T-cell based therapies will only work when there is a proper functioning HLA system present, which we have shown here to be the case.

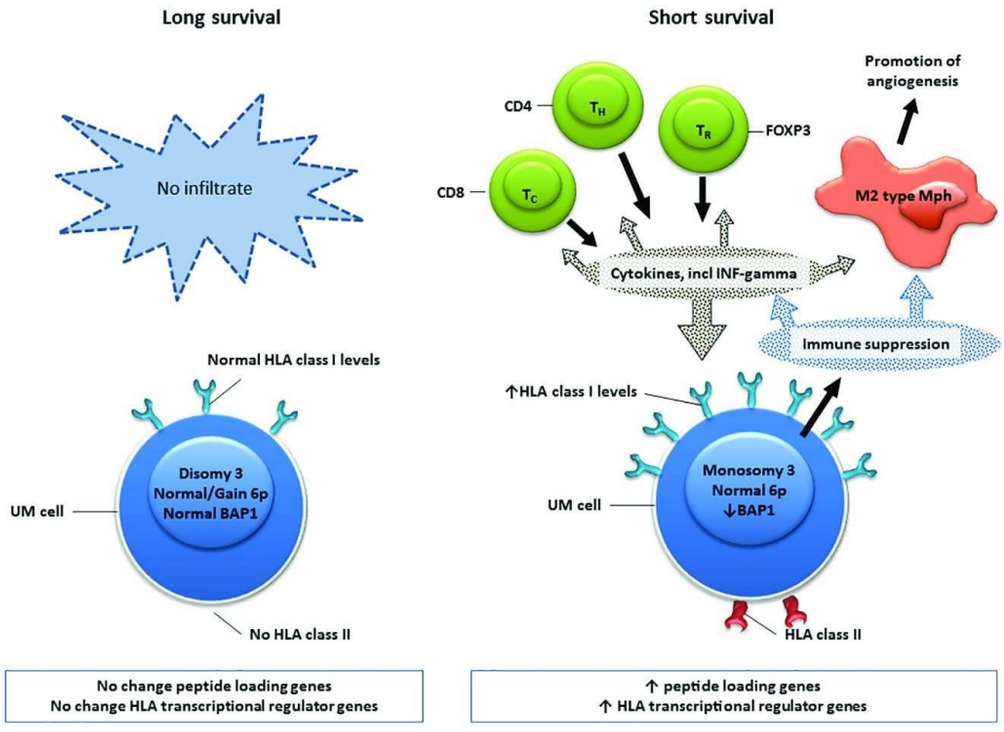


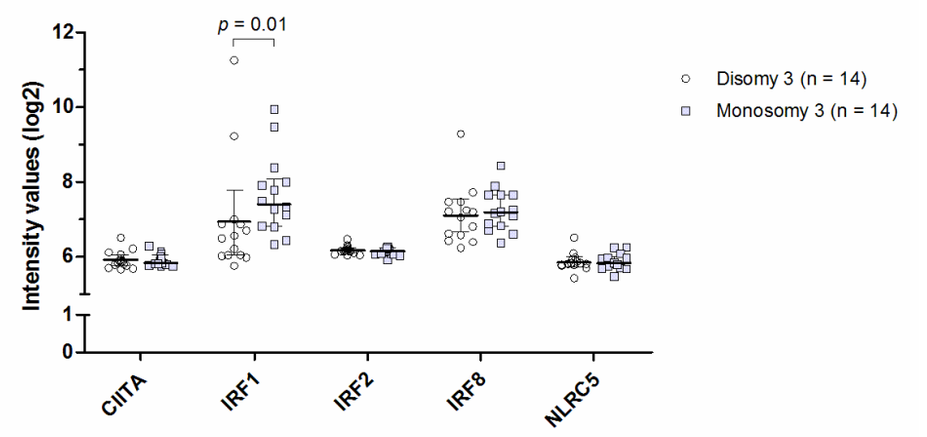
Figure 4. Schematic illustration of tumor characteristics and infiltrate. UM with monosomy 3 attract an infiltrate, producing different cytokines, including Interferon-gamma. The tumor cell (UM cell) responds by increasing HLA class I and II levels, as well as rendering the infiltrating immune cells ineffective (immune suppression) and creating a tumor-favorable environment, with amongst others, stimulation of angiogenesis.

In summary, we show that in primary UM, there is no gene-dose effect of chromosome 6p with regard to HLA expression nor a general abnormal internal regulation of HLA expression. We provide evidence for a co-regulated upregulation of genes encoding for HLA transcrip tional regulators, peptide-loading machinery, and HLA proteins on the cell surface, which we attribute to the presence of tumor-infiltrating T cells, secreting IFNG (Fig 4). This indicates a proper function antigen presenting system, which one would not expect, as likely the most common method a tumor deploys for immune evasion is reducing their antigen expression by down regulation of HLA molecules,[57,58] or altering the subtypes of HLA molecules expressed.[59] In earlier work we also showed that this latter is not the case.[30] Together, our data indicates that UM contain a proper functioning HLA antigen expression system, which should allow T-cell mediated tumor cell killing.

Acknowledgments

We like to thank W.G.M. Kroes, MSc, and C. Ruivenkamp, PhD, of the Center for Human and Clinical Genetics, LUMC, for their much appreciated work and valuable expertise on performing the SNP arrays.

Supporting Information



S1 Figure. Chromosome 3 status and gene-expression (Illumina array data) of several HLA regulators. Only significant p-values are shown, all other comparisons between the groups were not significant (p-values not shown). Error-bars represent the interquartile range. Results were obtained using the Mann-Whitney U tests.

S1 Table. An overview of the primers used for the validation with qPCR.

Primers	Forward	Reverse
Beta-Actin	CGGGACCTGACTGACTACCTC	CTCCTTAATGTCACGCACGATTTC
GAPDH	GCACAGCCCACAGGTTTC	CAGGCGGAGGACAGGATG
RPL13	GTACGCTGTGAAGGCATCAAC	GGTTGGTGTTCATCCGCTTG
RPS11	AAGCAGCCGACCATCTTTCA	CGGGAGCTTCTCCTTGCC
HLA-A	TGTGTTCGTGTAGGCATA	TTGAGACAGAGATGGAGAC
HLA-B	CTCCATCTCTGTCTCAACTT	CATCAACCTCTCATAGCA
B2M	TGCTGTCTCCATGTTTGATGTATCT	TCTCTGCTCCCCACCTCTAAGT
HLA-DR	CAAAGAAGGAGACGGTCTGG	GGCTCTCTCAGTTCCACAGG
HLA-DQ	TGATGGAGATGAGCAGTTC	GCAGCGGTAGAGTTGTAG
CIITA(-PIII)	GCTGGGATTCCTACACAATGC	TCTCCAGCCAGGTCCATCTG
IRF1	TCACCAAGAACCAGAGAA	TCCATCAGAGAAGGTATCAG
NLRC5	CTGGAGGAGTTGATGCTT	GATGGCTGAATGGTAGGT
TAP1	CGAAGCCCAGAAGTTTAG	CCACCAATGTAGAGGATTC
TAP2	CTATTCTGGTCGTGTGATTG	CTGTCTTAGTCTCCTGGAA

S2 Table. Percentage of tumor cells staining positive for the monoclonal antibodies for HLA-A, HLA-B or HLA-DR.

Tumor nr.	mAb HCA2 (HLA-A)	mAb HC10 (HLA-B/C)	mAb Tal.1B5 (HLA-DR)
01-042	0	5	10
01-074	40	25	60
01-091	50	60	10
01-129	80	100	30
01-131	50	60	20
02-158	0	0	5
02-167	70	90	15
02-174	20	70	5
02-199	30	10	15
03-086	30	5	25
03-087	5	0	5
03-120	50	70	100
03-129	60	5	10
04-018	20	10	15
04-035	80	70	15
04-074	5	5	10
04-075	60	50	25
04-103	20	10	5
04-112	50	40	10
20-005	90	60	10
20-042	25	25	5
20-125	10	0	5
20-128	50	40	50
20-173	40	5	80
20-178	25	10	35
99-184	5	10	20
99-187	5	0	10
99-239	95	80	30

S3 Table. Hazard ratio's (Univariate Cox-regression) of death due to metastasis for HLA gene expression as determined by the Illumina array.

Gene-expression array log2 intensity values (n=28)	Mean (median)	±SD	HR (*p*-value)	95% conf. interval
HLA-A	11.3 (11.4)	0.8	2.7 (0.003)	1.4-5.2
HLA-B	10.7 (10.8)	1.2	1.7 (0.004)	1.2-2.4
B2M	12.2 (12.2)	0.9	2.1 (0.01)	1.2-3.7
HLA-DR	9.0 (8.9)	0.9	1.4 (0.16)	0.9-2.4
HLA-DQ	7.3 (7.1)	1.0	1.3 (0.21)	0.9-1.9

HR = hazard ratio

S4 Table. Characteristiscs of study population, with hazard ratio's (HR) for death due to metastasis.

	Baseline Data				**Associations with gene-expr. (mean)**				
Categorical variables	**N**	**%**	**HR (*p*-value)**	**95% conf. interval**	***HLA-A***	***HLA-B***	***B2M***	***HLA-DR***	***HLA-DQ***
Patient gender									
Male	15	54	0.7 (0.60)	0.3-2.2	11.3	10.8	12.1	9.1.	7.5
Female	13	46	1.3 (0.60)	0.4-4.0	11.3	10.7	12.3	8.8	7.1
p-values					*1.00*	*0.72*	*0.50*	*0.47*	*0.41*
Prognostic groups									
Stage I & IIA	8	28	-		11.1	10.6	11.9	8.9	7.3
Stage IIB	10	36	4.4 (0.18)	0.5-30.6	11.3	10.6	12.2	9.0	7.3
Stage IIIA	8	29	14.1 (0.01)	1.7-117.9	11.6	11.3	12.7	9.1	7.2
Stage IIIB	2	7	5.1 (0.25)	0.3-82.8	11.1	10.2	12.0	9.2	7.7
p-values					*0.33*	*0.43*	*0.25*	*0.76*	*0.60*
Cell type									
Spindle	10	36	0.2 (0.44)	01-1.0	11.0	10.3	11.9	8.9	7.2
Mixed/Epithelioid	18	64	4.7 (0.44)	1.0-21.6	11.5	11.0	12.4	9.0	7.4
p-values					*0.09*	*0.13*	*0.21*	*0.91*	*0.83*
Chromosome 3									
Normal	14	50	0.04 (0.003)	0.01-0.35	10.1	10.1	11.8	8.9	7.2
Loss	14	50	22.6 (0.003)	2.9-176.1	11.7	11.4	12.6	9.1	7.4
p-values					*0.002*	*0.001*	*0.01*	*0.35*	*0.23*

Chromosome 6p (HLA loci)									
Normal	20	71	6.0 (0.09)	0.8-46.4	11.4	11.0	12.4	9.1	7.4
Gain	8	29	0.2 (0.09)	0.02-1.3	11.2	10.1	11.7	8.7	7.0
p-values					*0.47*	*0.049*	*0.09*	*0.38*	*0.47*
					Associations with gene-expr. (*p*-value)				
Numerical variables	**Mean (median)**	**±SD**	**HR (*p*-value)**	**95% conf. interval**	***HLA-A***	***HLA-B***	***B2M***	***HLA-DR***	***HLA-DQ***
Age at enucleation (years) (n=28)	62 (68)	15	1.0 (0.26)	1.0-1.1	0.046	0.008	0.001	0.02	0.15
LBD (mm) (n=28)	14 (15)	3	1.4 (0.01)	1.1-1.7	0.03	0.04	0.01	0.02	0.01
Prominence (mm) (n=28)	7 (7)	3	1.1 (0.58)	0.9-1.3	0.99	0.62	0.77	0.86	0.89

Baseline characteristics of patients and histological of 28 cases of primary UM, obtained by enucleation. The gene expression values of the indicated genes were determined by the Illumina array.
Tumor size (Largest Basal Diameter and Prominence) has been an important prognostic factor for a long time.
LBD = Largest Basal Diameter; HR = hazard ratio (Univariate cox regression).
Wilcoxon rank sum test (Mann-Whitney U) was used for comparing two categories, and the Kruskal Wallis Test was used for comparing more than two categories. *P*-values for associations with numerical data were calculated with Spearman's correlation (two tailed).

S5 Table. qPCR confirmation of the correlations found with the Illumina data, between expression values of HLA regulator genes and TAP1/TAP2, with HLA genes.

	HLA-A		*HLA-B*		*B2M*		*HLA-DR*		*HLA-DQ*	
HLA transcriptional regulators	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
CIITA	.437	0.02	.626	<0.001	.461	0.02	.615	<0.001	.430	0.02
IRF1	.527	0.001	.440	0.02	.591	0.001	.819	<0.001	.601	0.001
NLRC5	.331	0.09	.177	0.37	.363	0.06	.544	0.003	.539	0.003

	HLA-A		*HLA-B*		*B2M*	
Peptide loading machinery	*r*	*p*	*r*	*p*	*r*	*p*
TAP1	.824	<0.001	.744	<0.001	.878	<0.001
TAP2	.537	0.003	.446	0.02	.536	0.003

Underlined values confirmed the IIllumina-array results. *r* = two-tailed Spearman correlation coefficient. *p* = p-value.

S6 Table. Changes in mean expression of gene expression levels for markers identifying infiltrating cells, HLA genes, HLA regulator genes, peptide-loading machinery genes, and cytokines.

Marker	*Human tumor*	*Xenograft*	*Delta Human tumor-Xenograft*	*SD Human tumors*	*SD xenografts*	*P-values*
CD3D	6.39	5.05	1.35	1.12	0.22	< 0.001
CD3E	5.84	4.57	1.27	1.07	0.15	< 0.001
CD4	7.14	5.90	1.24	0.75	1.09	< 0.001
CD8A	7.52	6.61	0.91	0.84	0.16	0.001
CD8B	5.30	5.17	0.13	0.26	0.11	0.34
CD68	10.62	9.22	1.39	0.60	1.05	< 0.001
CD163	6.08	4.60	1.48	0.75	0.14	< 0.001
HLA-A	11.54	10.54	1.0	0.59	1.15	0.005
HLA-B	12.44	10.45	1.99	0.77	1.76	< 0.001
B2M	10.98	9.68	1.30	0.72	0.92	0.004
HLA-DRA	10.44	4.94	5.50	1.45	0.15	< 0.001
HLA-DQA1	7.25	4.18	3.07	2.09	0.19	< 0.001
HLA-DQA2	5.41	4.82	0.59	0.41	0.23	0.002
IRF1	7.61	6.38	1.23	0.87	0.23	< 0.001
IRF2	9.21	8.85	0.36	0.37	0.38	0.046
IRF8	7.09	5.43	1.66	0.84	0.17	< 0.001
CIITA	7.46	6.24	1.22	0.77	0.22	< 0.001
NLRC5	7.80	6.89	0.90	0.67	0.36	0.002
PDIA3	7.02	7.03	-0.01	0.25	0.22	0.93
TAP1	10.13	8.76	1.36	1.04	0.99	0.005
TAP2	8.88	8.13	0.75	0.74	0.52	0.02
Tapasin	10.30	9.58	0.72	0.49	0.56	0.004
Calreticulin	10.78	10.83	-0.05	0.16	0.17	0.54
IFNG	4.11	3.85	0.26	0.29	0.11	0.005
CCL2	7.87	5.93	1.94	1.33	0.81	< 0.001
TGFB1	7.84	6.91	0.92	0.54	0.63	< 0.001
TGFB2	6.96	6.77	0.19	0.78	1.41	0.14
TGFB3	7.04	6.55	0.49	0.77	0.41	0.08
TNF	5.79	5.71	0.08	0.35	0.17	0.40

The gene-expression was determined with the Affymetrix array in 12 primary human uveal melanoma/metastases and their corresponding xenograft in a mouse.

P-values have been calculated with the Wilcoxon-signed rank test.

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CHAPTER 7

Prognostic parameters in uveal melanoma and their association with BAP1 expression

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Abstract

Aim: To determine whether BAP1 gene and protein expression associates with different prognostic parameters in uveal melanoma and whether BAP1 expression correctly identifies patients as being at risk for metastases, following enucleation of the primary tumour.

Methods: Thirty cases of uveal melanoma obtained by enucleation between 1999 and 2004 were analysed for a variety of prognostic markers, including histological characteristics, chromosome aberrations obtained by fluorescence in situ hybridisation (FISH) and single nucleotide polymorphism (SNP) analysis and gene expression profiling. These parameters were compared with BAP1 gene expression and BAP1 immunostaining.

Results: The presence of monosomy of chromosome 3 as identified by the different chromosome 3 tests showed significantly increased HRs (FISH on isolated nuclei cut-off 30%: HR 11.6, p=0.002; SNP analysis: HR 20.3, p=0.004) for death due to metastasis. The gene expression profile class 2, based on the 15-gene expression profile, similarly provided a significantly increased HR for a poor outcome (HR 8.5, p=0.005). Lower BAP1 gene expression and negative BAP1 immunostaining (50% of 28 tumours were immunonegative) were both associated with these markers for prognostication: FISH cut-off 30% monosomy 3 (BAP1 gene expression: p=0.037; BAP1 immunostaining: p=0.001), SNP-monosomy 3 (BAP1 gene expression: p=0.008; BAP1 immunostaining: p=0.002) and class 2 profile (BAP1 gene expression: p<0.001; BAP1 immunostaining: p=0.001) and were themselves associated with an increased risk of death due to metastasis (BAP1 gene expression dichotomised: HR 8.7, p=0.006; BAP1 immunostaining: HR 4.0, p=0.010).

Conclusions: Loss of BAP1 expression associated well with all of the methods currently used for prognostication and was itself predictive of death due to metastasis in uveal melanoma after enucleation, thereby emphasising the importance of further research on the role of BAP1 in uveal melanoma.

Introduction

Uveal melanoma is a rare primary malignancy of the eye. Up to 50% of the patients may develop metastases, which are fatal in almost all cases.[1] A correct risk assessment is necessary in order to effectively select patients for inclusion in clinical trials, now that more effective drugs are being developed. An analysis of 8033 uveal melanomas showed a 10-year metastasis rate of 12% for small tumours (up to 3 mm thick) and 49% for large tumours (>8 mm thick).[2] It is therefore especially important to properly assess this risk in individuals with large tumours, such as those that need enucleation. Prognostic factors include histological factors such as cell type, involvement of the ciliary body, extrascleral extension and several chromosomal aberrations.[1] The parameter size, ciliary body involvement and extrascleral extension are often combined into one parameter used in the TNM classification, which provides a better prognostic value than any of these parameters individually.[3] Different techniques such as fluorescence in situ hybridisation (FISH) on tumour sections or isolated nuclei, or chromosome analysis techniques can be used to assess the tumour's chromosome status.[4–6] originally, loss of one chromosome 3 was identified as an important marker of poor prognosis, and this has been substantiated in many studies. However, later studies have also identified the importance of other chromosomes: gain of chromosome 8q is also correlated with death due to metastases,[5–14] while an extra chromosome 6p is associated with a better survival.[8,10, 15–18] In separate studies, gene expression profiling has also been identified as a reliable method for prognostication.[19, 20]

The pathophysiology of the importance of chromosome 3 loss was demonstrated by Harbour et al, who observed that loss of one copy of chromosome 3 together with inactivating mutations in the metastasis-suppressor gene encoding for BRCA1-asssociated protein 1 (BAP1) on the remaining copy of chromosome 3 was associated with the development of metastases. BAP1 is a deubiquitinating enzyme that is a member of the polycomb group proteins of transcriptional repressors and exhibits tumour suppressive activity.[21–23] Inactivation of BAP1 at the chromosome level may be the driving force for the development of metastases, and BAP1 levels may therefore influence survival.[7, 24–26]

For early detection of metastases and for studies on adjuvant treatment, it is important to know which techniques accurately predict the patient's prognosis. As loss of chromosome 3 is an essential step in the inactivation of BAP1,[27] we decided to compare BAP1 gene and protein expression in 30 cases of enucleated uveal melanoma of at least 8-year follow-up, with the results of a variety of techniques assessing chromosome aberrations, and with gene expression profiling based on the 15-gene classification assay described by Onken et al.[20, 24, 28]

Materials and Methods

Study population

Fresh-frozen material and formalin-fixed, paraffin-embedded specimens were obtained from the 30 uveal melanoma of which enough frozen material was left and good quality DNA was available. All 30 uveal melanomas had been enucleated at the Leiden University Medical Center (LUMC), Leiden, The Netherlands, between 1999 and 2004. Following enucleation, fresh tumour material was obtained immediately after the bulbus had been opened. Survival data and information on cause of death were obtained from the patient's charts and from the Dutch National Registry, and updated in November 2013. Each tumour sample was processed for conventional histopathological evaluation, including cell-type assessment according to the modified Callender classification at that time.29 The collection of material for research had been agreed upon by the Medical Ethics Committee of the LUMC and the research protocol adhered to Dutch law and the current version of the tenets of the Declaration of Helsinki (World Medical Association of Declaration 1964; ethical principles for medical research involving human subjects).

Chromosome aberrations and gene expression

Three different techniques were applied to determine the presence of aberrations of chromosomes 3 and 8: FISH on isolated nuclei (for chromosome 3) and single nucleotide polymorphism (SNP) analysis. FISH analysis on isolated nuclei was performed as described before.[30, 31]

DNA and RNA were isolated from fresh-frozen tissue. DNA for SNP analysis was extracted with the QIAmp DNA Mini kit and RNA for gene expression profiling with the RNeasy mini Kit (both from Qiagen, Venlo, the Netherlands). SNP analysis was performed with the Affymetrix 250K_NSP microarray chip (Affymetrix, Santa Clara, California, USA) on all 30 uveal melanomas. Gene expression analysis on BAP1 was carried out on RNA of 28 tumours using the Illumina HT-12v4 chip (Illumina, San Diego, California, USA). RNA obtained from frozen material from all 30 uveal melanomas was tested in the 15-gene classification assay as described by Onken et al.[28] and results sent to the Department of Ophthalmology and Visual Sciences of Washington University School of Medicine (St. Louis, Missouri, USA) for class assignment.

BAP1 immunohistochemistry

Immunohistochemical staining was performed for 28 patients from whom enough tumour material was available using the Ventana Benchmark ULTRA fully automated staining system (Ventana Medical Systems Inc, Tucson, Arizona, USA) with an alkaline phosphatase red detection kit.[32] In short, sections were deparaffinised and then heated using Heat-induced Epitope Retrieval for 64 min at 97C°. The sections were then incubated for 32 min at 37C° with the primary BAP1 antibody (sc-28383, concentration 1:50, Santa Cruz Biotechnology, Dallas, Texas, USA). Target amplification was

performed and then followed by incubation with haematoxylin II counterstain for 8 min. An additional counterstain was performed with blueing reagent (Ventana Medical Systems Inc.).
Liver, tonsil, breast and surrounding non-malignant tissue, as well as intratumoral vessels and inflammatory cells, were used as positive controls. As negative control sections without antibody were used.[32] Tumours were scored either negative or positive for the BAP1 nuclear staining.

Statistical analysis

For data analysis, we used the statistical programming language R V.3.0.1 (R: A Language and Environment for Statistical Computing, R Core Team, R foundation for Statistical Computing, Vienna, Austria, 2014, http://www.R-project.org) supplemented with specialised packages for SNP and RNA analysis. The main package used for SNP analysis was aroma.affymetrix,[33–35] supported by 'DNAcopy' (Venkatraman E. Seshan and Adam Olshen, DNAcopy: DNA copy number data analysis. R package V.1.34.0), 'sfit' (Henrik Bengtsson and Pratyaksha Wirapati (2013), sfit: Multidimensional simplex fitting. R package V.0.3.0/r185, http://R-Forge.R-project.org/projects/ matrixstats/) and 'R.utils' (Henrik Bengtsson (2014), R.utils: Various programming utilities, R package V.1.29.8, http:// CRAN.R-project.org/package=R.utils). As reference set, we used the data of 84 healthy controls obtained with the same Affymetrix 250K Nsp chip (Affymetrix, Santa Clara, California, USA) by the Department of Human Genetics at our centre. The 'Aroma.Affymetrix' package made it possible to use these SNP microarrays to determine copy number values.[33–35] The packages used for RNA microarray analysis were 'limma' V.3.16.836 and the specific packages for Illumina microarrays: 'lumi' V.2.12.0,37–40 'annotate' (R. Gentleman, annotate: Annotation for microarrays, R package V.1.38.0), and the database package 'illuminaHumanv4.db' (Mark Dunning, Andy Lynch and Matthew Eldridge, illuminaHumanv4.db: Illumina HumanHT12v4 annotation data (chip illuminaHumanv4), R package V.1.18.0).
Mann–Whitney U test for non-parametric analysis was used to assess differences in the amount of BAP1 gene expression, and $\chi 2$ tests for comparing the prognostic parameters with the BAP1 staining on immunohistochemistry (IHC). Univariate Cox regression was applied with events determined as 'death due to UM' and right censoring. Cases of which the cause of death was unknown were censored as well. To calculate the respective HRs of the different parameters, the BAP1 gene expression was dichotomised at the median to create two equal groups. All analyses were performed with SPSS V.20.0.1 (IBM SPSS Statistics, IBM Corporation, Armonk, New York, USA).

Results

Patients

We studied a group of 30 uveal melanoma patients, consisting of 13 men and 17 women with an average age at the time of enucleation of 61.7 years (range 28–84 years; median 66.5 years) and a mean follow-up of 77.5 months (range 14–155 months). Of these patients, 14 had died of uveal

melanoma metastases (mean survival 36.7 months; range 14–96 months) and 3 of unknown causes (mean survival 85.0 months; range 63–126 months), while no patient has been lost to follow-up. The mean largest basal tumour diameter was 13.7 mm (range 8–18 mm; median 13.5 mm), with an average prominence of 7.3 mm (range 2–12 mm; median 7.0 mm). The pathological TNM stages showed stage I in 4, stage IIA in 5, stage IIB in 10, stage IIIA in 9 and stage IIIB in 2 cases.41 The ciliary body was involved in 13 cases. The histological cell type was spindle in 11, mixed in 14 and epithelioid in 5 cases. Immunohistochemical staining for BAP1 (figure 1) was positive in 14 of the 28 patients who could be tested. BAP1 staining was either present on more than 95% of the tumour cells or almost completely absent. An overview of all variables per patient is available (see online supplementary table S1).

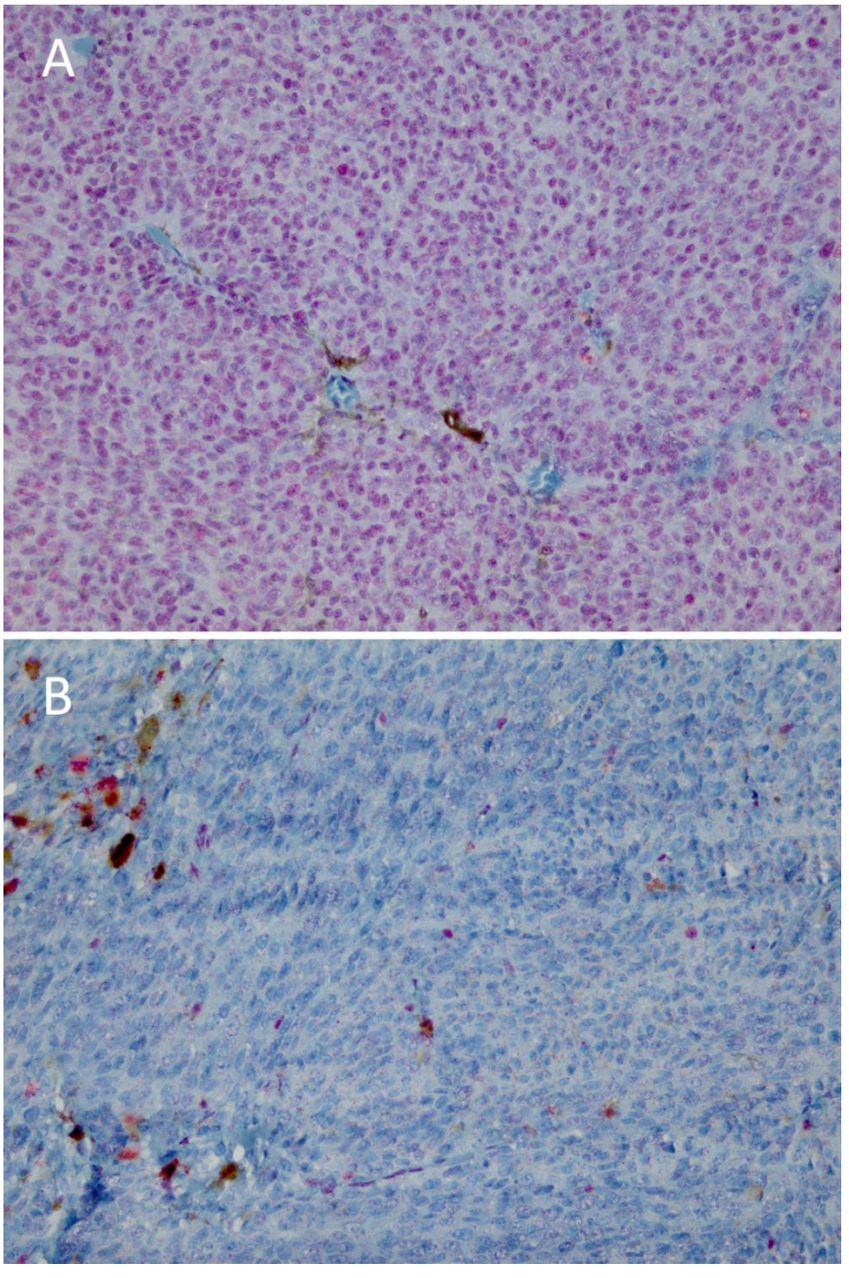

Figure 1. Examples of a BAP1-immunopositive (A) and a BAP1-immunonegative (B) tumour.

Table 1. Comparison of different prognostic parameters with survival in 30 uveal melanoma patients

		Univariate analysis – unknown cases attributed to death due to other causes			
			95% confidence interval		
Parameters	n	HR	Lower	Upper	p-value
Standard clinical/histological parameters					
Gender					
Male	17	-	-	-	-
Female	13	1.3	0.5	3.7	0.63
Ciliary Body involvement					
No	17	-	-	-	-
Yes	13	6.3	1.9	21.0	0.002
Stage group					
Stage I & IIA	9	-	-	-	-
Stage IIB	10	5.0	0.6	45.0	0.14
Stage IIIA	9	16.9	2.1	138.8	0.009
Stage IIIB	2	5.8	0.4	92.6	0.22
Age at enucleation (years; low to high; continuous)	30	1.0	1.0	1.1	0.26
Largest basal diameter (mm; low to high; continuous)	30	1.4	1.1	1.7	0.008
Chromosomal aberrations					
FISH on isolated nuclei, cut-off at 5%					
Disomy chromosome 3	11	-	-	-	-
Monosomy chromosome 3	19	12.1	1.6	93.2	0.017
FISH on isolated nuclei, cut-off at 30%					
Disomy chromosome 3	15	-	-	-	-
Monosomy chromosome 3	15	11.6	2.5	52.5	0.002
SNP on tumour DNA					
Disomy chromosome 3	14	-	-	-	-
Monosomy chromosome 3	16	20.3	2.6	156.7	0.004
Disomy chromosome 8q	11	-	-	-	-
Gain chromosome 8q	19	11.7	1.5	89.7	0.018
Disomy chrom 3 + disomy chrom 8q	10	-	-	-	-
Monosomy chrom 3 + gain chrom 8q	15	8.9	2.0	40.4	0.004
Gene expression					
15-gene expression assay class					
Class 1	14	-	-	-	-

Class 2	14	8.5	1.9	38.3	0.005
BAP1 gene expression (dichotomized at median)					
High	14	-	-	-	-
Low	14	8.7	1.9	39.7	0.006
BAP1 gene expression (high to low; continuous)	28	4.0	1.5	10.6	0.006
Immunohistochemistry					
Positive BAP1 immunostaining	14	-	-	-	-
Negative BAP1 immunostaining	14	5.5	1.5	20.1	0.010

An univariate Cox regression analysis was performed.

FISH, fluorescence in situ hybridization; SNP, single nucleotide polymorphism.

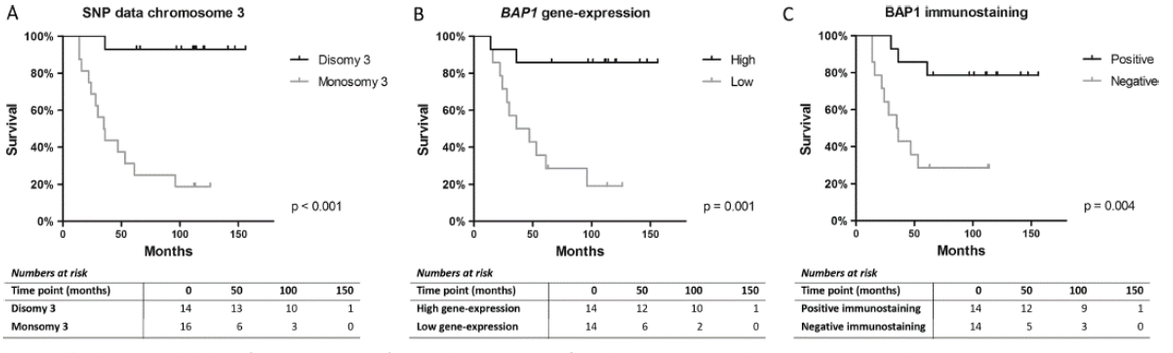

Figure 2. Kaplan–Meier (log-rank test) survival graphs for single nucleotide polymorphism data on chromosome 3 status (A), BAP1 gene expression dichotomised at the median (B), and for BAP1 negative and positive staining as seen on immunohistochemistry (C).

Chromosome aberrations

FISH analysis of chromosome 3 on isolated nuclei with a cut-off value of 5% indicated 19 cases of monosomy of chromosome 3, while a cut-off value of 30% identified 15 cases.

SNP analysis revealed 16 cases with monosomy of chromosome 3, and one case with a partial deletion from 3q28 till the end of the chromosome. With this technique, 15 out of the 16 monosomy 3 tumours had a gain of the long arm of chromosome 8 (8q) compared with four of the disomy 3 tumours.

Gene expression profiling

The 15-gene expression assay of Onken et al, performed on all 30 tumours, classified 14 of the tumours as class 1 (good prognosis) and 16 as class 2 (poor prognosis) uveal melanoma. **Associations**

A univariate Cox regression analysis was performed to compare the impact of all clinical, histological, chromosomal and gene expression data on death due to metastases. All categorical variables fulfilled the proportional hazards assumption. All parameters, except gender and age at enucleation, were associated with increased risk of death due to metastasis, with the chromosomal aberration

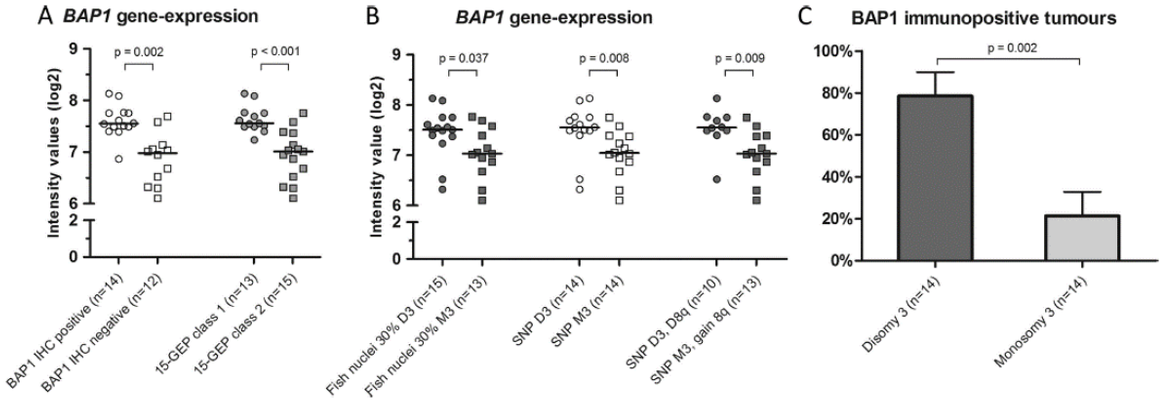

Figure 3. Median BAP1 gene expression compared with the immunohistochemistry of BAP1 and different parameters used for prognostication. (A, B), and a bar graph showing the distribution of BAP1 immunopositive tumours with regards to disomy and monosomy of chromosome 3 (C).

p Values were calculated with Mann–Whitney U test and corrected for multiple comparisons (Benjamini–Hochberg; n=7) for A and B, and with the χ2 test for C. GEP, gene expression profiling; D3, disomy of chromosome 3; M3, monosomy of chromosome 3; D8q, disomy of chromosome 8q.

s analysed with SNP, and the stage groups having relatively wide CIs (table 1). When the cases for which the cause of death was unknown were attributed to death due to metastasis, the results were similar (see online supplementary table S2). Multivariate analysis of all parameters led to results containing one in the 95% CI, or having infinite as upper limit, or both (data not shown).

Loss of chromosome 3 was associated with death due to metastasis (figure 2A). Dividing the tumors in two groups based on chromsome 3 and 8q (either both disomic or both altered: loss of chromosome 3 together 8q gain), as determined by SNP analysis, associated with the 15-gene expression classes of Harbour (χ2 test $p<0.001$; one cell, had an expected count less than 5).

The Kaplan–Meier survival graphs showed a discriminative function for BAP1 gene expression and the BAP1 immunostainings with regards to death due to metastasis (figure 2B, C).

Low BAP1 gene expression (RNA) was associated with lack of immunohistochemical staining of BAP1 on uveal melanoma cells ($p<0.001$ for dichotomised data, χ2 test; $p<0.001$ for continuous data, independent t test). When the RNA expression values of BAP1 were compared with the 15-gene expression assay classes, and the different methods assessing chromosome 3 aberrations, significant associations were seen for all comparisons, with lower RNA values for the gene expression class and the aberrations that were associated with poor prognosis (figure 3A, B). Especially the strong association between the RNA values of BAP1 and the 15 gene expression profile is striking.

The BAP1 immunostaining corresponded to the expected Harbour gene expression class in 23 of the 28 cases. Similar to the findings at the RNA level, loss of chromosome 3 as seen by the two different methods was associated with a negative BAP1 immunostaining (figure 3C).

Discussion

The different methods that identify monosomy of chromosome 3 as well as the gene expression-based classifications had increased HRs for death due to metastasis. These parameters were all associated with lower RNA levels of BAP1 and negative immunohistochemical staining for BAP1, and moreover, these BAP1 expression levels themselves were predictive for death due to metastasis of uveal melanoma (p=0.01).

Harbour et al.[27] identified an important role in the development of metastases in uveal melanomas for a specific gene on chromosome 3, that is, BAP1, and suggested that loss of one copy of chromosome 3 may unmask inactivating mutations in the metastasis-suppressor gene BAP1 on the remaining copy of chromosome 3. We were able to classify uveal melanoma easily into two groups using gene expression profiling (15-gene expression profile classification), as has already previously been shown by Tschentscher, Onken and van Gils.[19, 20, 42] In our study, the loss of one copy of chromosome 3 together with gain of chromosome 8q was highly correlated to the 15 gene expression profile class 2, while we had only enough tumour material to perform a gene expression analysis in 28 of the 30 tumours and our number of samples was relatively small.

Interestingly, one case was staged as stage IIA and still alive after 9 years' follow-up, despite having a monosomy of chromosome 3 together with gain of chromosome 8q and being a class 2 tumour (15-gene expression assay). In this case, the BAP1 immunostaining scored negative as well.

In large tumours, strong positive associations have been described between the prognostically poor 15-gene expression profile known as class 2 and the presence of epithelioid cells, the extracellular matrix pattern known as networks and largest basal tumour diameter, all of which associations have also been described to be related to monosomy of chromosome 3.[19, 20, 43, 44] As we observed an almost perfect association between the presence of monosomy of chromosome 3 plus 8q gain and the 15 gene expression profile class 2, we hypothesised that both should associate with loss of BAP1. We indeed noted a significant association for lower BAP1 gene expression (dichotomised and continuous data) and negative BAP1 immunostaining, with the combined presence of monosomy of chromosome 3 and chromosome 8q gain, as well as with the 15 gene expression prolife class 2. This is identical to the results published by Harbour et al.[27]

Our data show that BAP1 gene expression correlated with the findings of the BAP1 immunohistochemistry. Previously Harbour et al.[27] showed this for six tumour samples. The association between RNA and immunohistochemistry suggests that a cut-off value of the BAP1 gene expression, for example, measured with a quantitative PCR, could be made to predict loss of BAP1 immunoreaction. Shah et al.[45] and members of our group[32] have shown that immunohistochemistry

for BAP1 protein expression might be an easy way to discriminate between long and short survival, and may even replace mutation analysis of BAP1 in uveal melanoma patients. In large uveal melanoma, loss of one copy of chromosome 3, together with gain of chromosome 8q, clearly leads to this specific gene expression profile known as class 2, which is associated with loss of BAP1 gene expression, and with metastases formation.[20, 27] This loss of BAP1 plays an important role in developing malignant tumour behaviour,[27] and it may also be involved in the development of an inflammatory phenotype, as this was previously found to be associated with monosomy of chromosome 3.[46] Although Harbour's group originally reported a strong association between the presence of the 15-gene expression profile class 2 and monosomy of chromosome 3, they stated in their most recent papers that monosomy 3 is not a good prognostic marker.[47] This was based on the use of different tests on material obtained from enucleated uveal melanoma as well as on biopsies.[47] However, the small amount of material obtained from biopsies may not have been sufficient for the specific SNP assay that they used, or small tumours may not represent the same profile in all parts of the tumours.

Now that several prognostication techniques have been developed that work accurately in the highest risk patients, that is, those that undergo enucleation, the next challenge is to determine the exact way how these inactivation mutations in BAP1 lead to metastasis formation.

In summary, our results show that monosomy 3/8q gain and the class 2 gene expression profile are both highly associated with lower BAP1 gene expression and negative BAP1 immunostaining, and that both methods for assessing BAP1 levels are predictive for death due to metastasis in uveal melanoma after enucleation. This emphasises the importance of further research on the role of BAP1 in the development of the inflammatory phenotype and the pathophysiology of the role of BAP1 in metastasis formation in uveal melanoma.

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CHAPTER 8

Summary and General Discussion

Summary and General Discussion

Ocular Immunology

The immunological characteristics of the eye are intriguing and characterized by immune-privilege and immune deviation. Because of its avascularity, the cornea has already a higher threshold before immune responses take place. Directly behind the cornea is the anterior chamber where immune responses are influenced by an active system of immunosuppression, known as ACAID (Anterior Chamber Associated Immune Deviation).[1] This immunoregulatory system raises an additional threshold for any immune response. In this complex system, we aim to place the artificial cornea made out of fish collagen, which should ideally not elicit an immune response.

Fish Scale-derived Collagen Matrix

The Fish Scale-derived Collagen Matrix (FSCM) represents a new approach to replace human donor corneas. We need an alternative to human donor corneas as there is a worldwide donor shortage, even in the developed world. This is tragic, as cornea transplantation is the most successful form of organ transplantation, largely attributable to the immune privilege that the cornea exhibits.[2, 3]

The make-up of the FSCM is similar to the biosynthetic artificial cornea, used already in a phase one clinical study, with respect to being an acellular collagen scaffold.[4] The FSCM differs by being of natural (fish) origin and not being artificially constructed: its natural origin should ideally lead to a cheaper fabrication process. The challenge is to keep the modification process after harvesting the scales as simple and straightforward as possible. This could give some difficulties as the FSCM differs from batch to batch and also individually, as every scale has different dimensions and is not uniform with regard to thickness. However, technical modifications may help to overcome this.

The most well-known alternative to normal human donor cornea transplantation is the Boston Keratoprosthesis (Kpro),[5] which is used in cases where regular cornea transplantation would not suffice. The Boston Kpro consists of a central optical cylinder, a titanium back plate and a locking ring, with a donor cornea in the middle. This Boston Kpro is a good solution for cases where cornea transplantations have failed, however, it does not reduce the shortage of human donor corneas, as it still needs a donor cornea sandwiched between the optical cylinder and the back plate. Alternatives to the donor cornea for the Boston Kpro are being investigated.[6] The FSCM has the features needed to serve as an alternative for human donor corneas. This collagen scaffold has the same arrangement of collagen fibers, although a bit denser, and facilitates the regrowth of human corneal epithelial cells and adhesion of keratocytes (corneal fibroblasts) in vitro. The regrowth of corneal epithelial cells is comparable for human epithelial cell lines or primary cultured human corneal epithelial cells. The anterior surface of the initial FSCM is not smooth; it exhibits a micropattern that differs from location to location. Yet the growth of human corneal epithelial cells is not hampered by these different surfaces. The keratocytes adhere to the FSCM, which we proved by the positive staining for integrin-α6 and –β1. No infiltration of keratocytes was observed during the relatively short follow-up periods

(maximal 2.5 months). The results were similar to those with the biosynthetic artificial cornea used in a phase 1 clinical trial, where the first signs of keratocyte immigration were seen after 6 months at the skirts, anchoring it in place, and where signs of repopulation were seen in 7 out of 10 patients at 12 months.[4]

The FSMC is highly permeable for oxygen and glucose, at an even higher permeability rate than the human cornea.[7, 8] This is important in order to maintain a vital epithelial layer. The technical aspects allow suturing of the FSCM into place into the recipient corneal bed. As with all ocular tissues, subtle handling is advised, as the FSCM can tear upon too brisk manipulation. This could prove itself a cumbersome threshold to overcome, as tearing during surgery is a serious problem, needing the FSCM to be replaced. The FSCM is a bit tougher than the human cornea, which provides advantages and disadvantages. One of the advantages is that the curvature of the FSCM will be maintained, however, its disadvantage is that a reasonable adequate curvature with regard to refraction should already be achieved during the production process. The smoothness of the FSCM's rim depends on the cutting procedure; in case of irregularities at the edge, a softer FSCM is more forgiving then a brisk version.

The FSCM facilitates a similar direct light transmission in the visible spectrum as the human cornea (90% versus 91%). This high rate of light transmission is not directly correlated with a good visual acuity, as for this the curvature and, to a lesser amount, the light scatter of the scaffold are important as well. The required curvature is individually based and furthermore largely influenced by sutures and in a lesser way by corneal swelling, which is also the case with human donor corneal transplants. The light scattering of the first versions of the FSCM with the pattern still on top was equal to early to moderate cataract with log(s) = 1.62. This does not necessarily influence visual acuity,[9, 10] but could produce some haze and lead to decreased contrast vision. Unpublished data of experiments using the FSCM with the micro-pattern removed, revealed improved light scatter values. As far as we know, no forward light scatter values for the biosynthetic artificial cornea have been published. The optical properties of Boston Kpro have been extensively investigated, but we could not find a comparison with the human cornea with regard to forward light scatter.[11]

Most importantly, our animal studies in which we implanted the FSCM in non-immunoprivileged locations show that the FSCM has an excellent biocompatibility, with similar immune responses as sham-surgery and no sensitization of the immune system upon subcutaneous implantation. When there is no specific immune response triggered when the FSCM is implanted subcutaneously, it is reasonable to expect that such an immune response is also absent in an avascular tissue such as the cornea.

Upon intra-stromal introduction into the cornea of rabbits, the FSCM maintained its transparency, and elicited neither corneal melting, neovascularization nor any immune response, during the six weeks we left it in place. This confirms that the FSCM transmits enough oxygen and nutrients to keep the overlaying epithelium vital and that the FSCM is biocompatible with the cornea. Studies on the biosynthetic artificial cornea showed already that repopulation by epithelial cells is feasible.[4]

There are a few features of the FSCM that require some adjustment and further research. We know that human corneal epithelial cells can repopulate the FSCM surface in vitro, but we need to confirm this in vivo. Are we going to use the FSCM for anterior lamellar keratoplasty only, thus leaving the recipient's corneal endothelium intact, or are we aiming for penetrating keratoplasty as well? Before we can make such decisions, we need to know first whether the FSCM and the overlaying epithelium remain stable without an endothelial layer on its posterior surface. Secondly, we need to know how strong the interaction is between the corneal fibroblast and the FSCM. Will the connection between the FSCM and the rest of the cornea be strong enough to compete with the insertion of a human donor cornea?

Long term animal-studies are needed to determine the long term compatibility of the FSCM, especially with regard to the stability of the scaffold and its interaction with the cornea fibroblasts

Currently, the research focusses on making the FSCM less brisk and to produce a smoother surface and on its use as an emergency patch at the time of corneal perforation. A clinical study to determine its applicability is ongoing.

Uveal Melanoma

While the ocular immune privilege allows acceptance of foreign tissue in the cornea, it also allows growth of intraocular tumors. We looked into the immunology of Uveal Melanoma (UM) because we want to control or cure this malignancy. The tumor is located in the ciliary body and choroid, which are richly vascularized, and lie outside the blood-retina-barrier. These tissues contain the rich networks of innate immune cells (bone marrow-derived resident macrophages and dendritic cells). These cells, together with the parenchymal cells, secrete immune mediators, that support immune-privilege.[12] Several studies show that the immune system is active at the tumor site, yet it is apparently not doing its job effectively in halting and destroying the tumor. We showed that higher HLA protein expression on the tumor surface is associated with shorter patient survival.[13] [14] It could be that certain HLA-alleles are predisposing for this, as we know for example that several autoimmune diseases have HLA-associations such as with HLA-B27, and there are specific regulators that influence the level of class I expression. However, our research revealed no association for a specific HLA-genotype predisposing to an increased HLA protein expression in uveal melanoma.

We know that in most tumors, HLA expression is downregulated or skewed towards the non-classical HLA types.[15] In contrast, in uveal melanoma, classical HLA expression is upregulated. What drives this increased HLA class I expression? Is this directly influenced by tumor genetics or indirectly by the immune infiltrate? Based on the analysis of data from 28 enucleated eyes with uveal melanoma, we found that there was no dosage effect of chromosome 6p, on which the genes encoding the HLA antigens are located. We confirmed the known association between a higher expression and monosomy of chromosome 3. The protein levels of the HLA Class I and II molecules were positively associated with their mRNA gene expression, which is to be expected. The genes encoding the

peptide-loading molecules had similarly raised levels. These peptide-loading molecules are needed and responsible for putting the peptides in the groove of the HLA molecules before they are presented on the cell's outer surface. The HLA molecules are not functional without them. Altogether, this indicates that expression of the HLA antigens is not impaired.

We confirmed that an increased amount of tumor-infiltrating immune cells was associated with increased levels of HLA on the tumor cell surface. This was as expected, based on previous research.[16] . What is new in uveal melanoma is that we show that when the human immune cells are absent, the amount of HLA allele expression subsequently indeed decreases. We demonstrated this by analyzing xenografts of the human tumor in immunodeficient female SCID mice. This finding proves our earlier statement that the increased HLA levels are not directly influenced by the genetics of the tumor, but that the HLA expression level is under the influence of the immunological triggers in its direct environment. We can therefore assume that in uveal melanoma the HLA-system responds normally to the input of the immune system and is not under direct influence of the tumor. Knowing this, the next question to be answered will be whether the tumor will be attacked and removed by the lymphocytes when implanted in mice with a patent immune system, or whether the uveal melanoma will still be able to create an environment which renders the mouse's immune system ineffective?

Which factors of the immune response in uveal melanoma are responsible for triggering the increased HLA expression? It could very well be that the increased HLA expression in uveal melanoma is under influence of interferon-gamma (IFNγ), as the tumor-infiltrating lymphocytes and macrophages produce IFNγ which activates HLA transcriptional regulators. [17, 18] IFNγ is predominantly secreted by CD4+ T helper 1 cells (Th1), CD8+ cytotoxic T cells, and NK cells, and to a lower degree by the professional antigen-presenting cells (APCs) including macrophages, and lastly also by B-cells.

We previously showed that IFNγ stimulation resulted in an increased expression of HLA Class I and Class II molecules in uveal melanoma cell lines.[19, 20] Those studies were done on a limited number of available cell lines, yet demonstrated that *in vitro* the HLA regulation is not aberrant. The UM cell lines during co-culture with IFNγ-secreting T cells, synthesize chemokines such as CXCL8-11, CCL2 and CCL5, VEGF and ICAM1 that create a tumor-promoting environment by attracting monocytes, including M2 type macrophages. M2 macrophages are known to promote angiogenesis.[21]

Normally, the presence of classical HLA-alleles together with infiltration of a tumor by immune cells should lead to the destruction of the tumor. In uveal melanoma, it does not. This could be due to an increase in PD-L1 levels in response to IFNγ exposure, which makes UM cells resistant to lysis by cytotoxic T-cells.[22] Overall, it is clear that the tumor's immune response is skewed towards angiogenesis and growth factors,[23] instead of tumor cell lysis.

A recent study treating metastatic uveal melanoma patients with an adoptive transfer of autologous tumor-infiltrating lymphocytes showed objective tumor regression in 35% of these patients. The

tumor infiltrating lymphocytes were selected based on tumor reactivity and subsequently expanded in cell culture, prior to their transfer.[24] This study demonstrates that tumor infiltrating lymphocytes do exist in metastases of uveal melanoma and can be triggered to act against their autologous tumor during in vitro culture, which leads to our hypothesis that the HLA alleles in uveal melanoma mostly, but not only, present peptides that are not recognized as pathogenic

Recently, Gezgin et al. showed that tumors with many infiltrating lymphocytes have a loss of BAP1 expression. In this thesis, we show that monosomy of chromosome 3 and the gene-expression profile class 2, are associated with loss of BAP1: [25] both prognostic factors are associated with a lower BAP1 gene expression and a negative BAP1 immunostaining. We also confirm that on its own, BAP1 gene-expression and BAP1 immunostaining are predictive of death due to metastasis. This was recently again confirmed by the TCGA study (2017). Gezgin's subsequent work shows that it is possible that BAP1 is an inflammatory regulator in uveal melanoma. It may be that BAP1 by itself promoted the secretion of regulators of a pro-angiogenesis environment, or that it promotes the influx of cytokine-producing T cells or macrophages.

Further research should be directed at finding peptides that are being presented as stimulators of anti-uveal melanoma T cells. Another option is to aimed at expanding possible therapies based at peptides that can be recognized such as PRAME, which has been demonstrated to be expressed in uveal melanoma.[26, 27] Another research focus could be aimed at counteracting the skewing of the immune response towards angiogenesis and growth factors.

Conclusion

We used the corneal immunosuppressive environment to test the possibility of inserting a Fish Scale-Derived Collagen Matrix in a corneal pocket, and the excellent results and lack of primary and secondary immune responses led to a clinical trial, which is currently underway.

The immunosuppressive environment of the eye allows the growth of malignant melanocytes which leads to the formation of uveal melanoma. The association between increased numbers of macrophages and lymphocytes in uveal melanoma and an increased development of uveal melanoma metastases suggest an influence of pro-angiogenic macrophages, The relation between loss of BAP1 expression and a very bad prognosis and the influx of leukocytes into the primary tumor, suggests that BAP1 functions as a regulator of inflammation. Further research will focus on the role of BAP1 in the regulation of inflammation in the uveal melanoma microenvironment.

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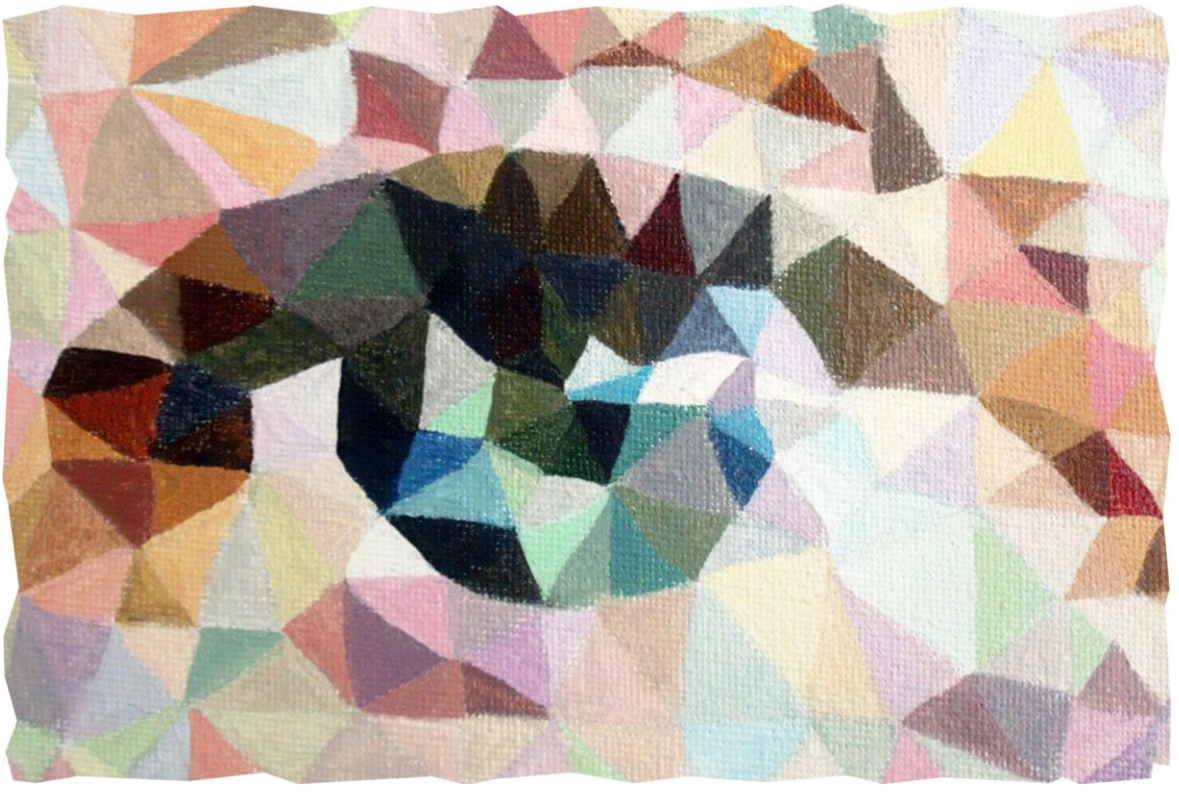

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Curriculum Vitae

Curriculum Vitae

Herbert van Essen was born on the 19th of January 1980 in Dordrecht, The Netherlands. He received his secondary school education at the Guido de Bres, in Rotterdam and in 1998 he went on to study *Computer Science and Engineering* at the Windesheim College in Zwolle. After graduating in 2002 and simultaneously passing the state exam in chemistry, the author filled an interim year studying philosophy at the University of Utrecht. In 2003, he began his medical training at the Leiden University Medical Center (LUMC). To keep up his earning, he kept a side-job as programmer at Tritac Systems, Leiden, from 2006-2009. In 2009, he completed his clinical rotations, among which an internship at a small hospital in a developing area in Raxaul, India. In the same year, he started a part-time job as a medical intern at the department of internal medicine at the Alrijne Hospital in Leiderdorp, which he combined with his first ophthalmological research on uveal melanoma under the supervision of Prof. Dr. Martine J. Jager at the LUMC. This formed the beginning of his full-time PhD training in 2010 which focused largely on a fish-scale derived artificial cornea, a collaboration between the LUMC and the biomedical company Aeon Astron Europe B.V., with a grant from the Department of Economic Affairs of the Dutch government. At the same time he continued his research on uveal melanoma. The author started his residency in Ophthalmology at the LUMC in 2014 under the supervision of Prof. Dr. Gre P.M. Luyten.

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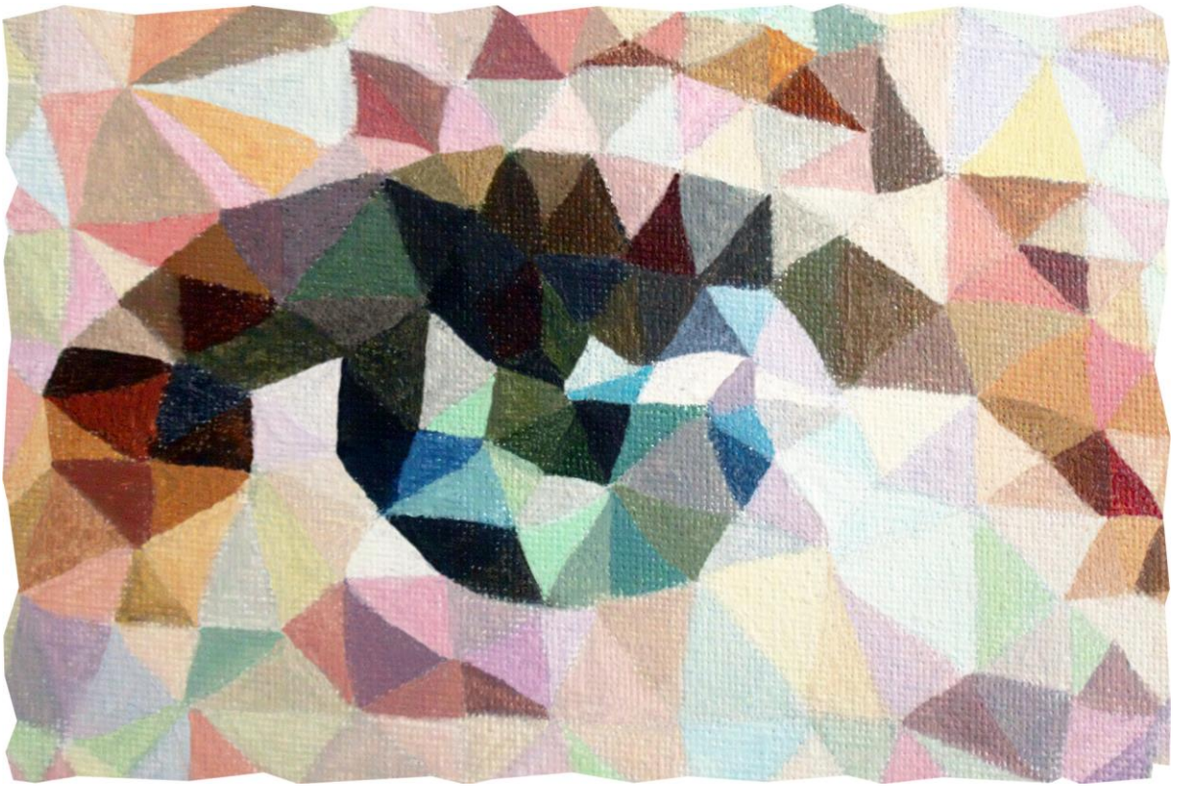

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