Ocular responses to foreign corneal and tumor issue

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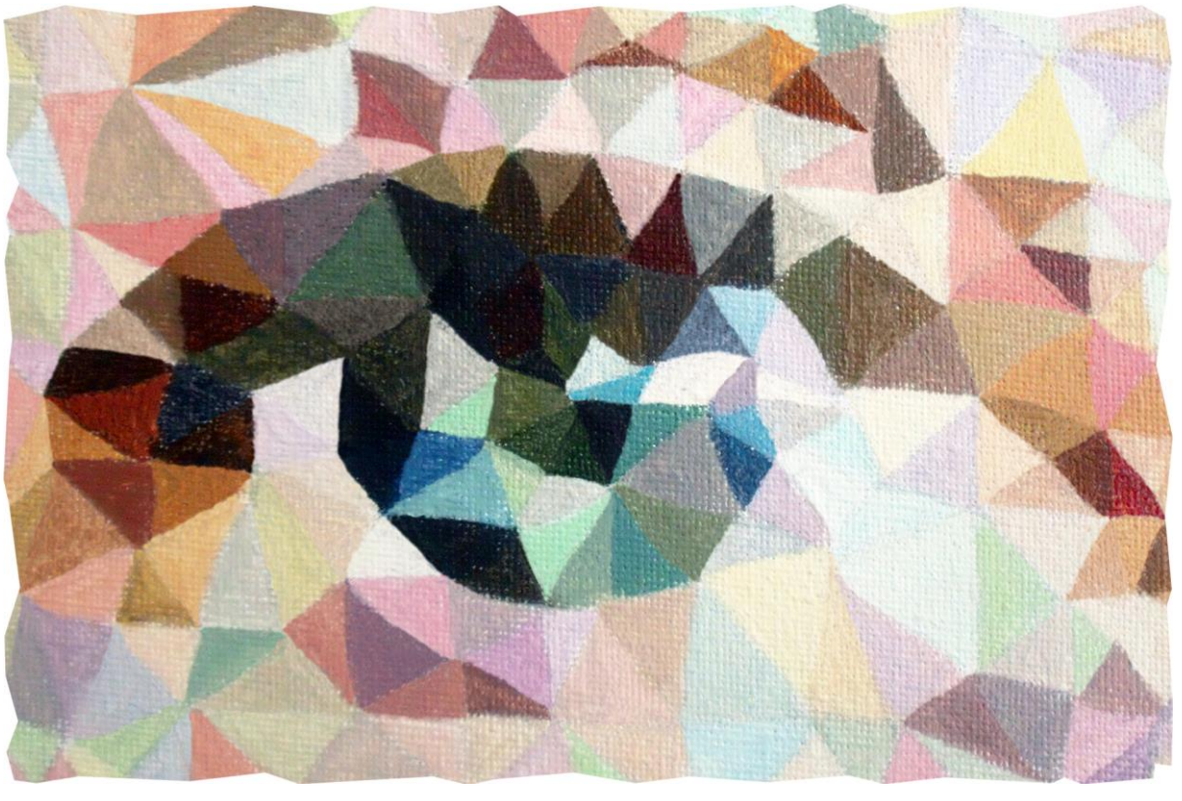
CHAPTER 1

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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