Inflammation and immunomodulation in uveal melanoma

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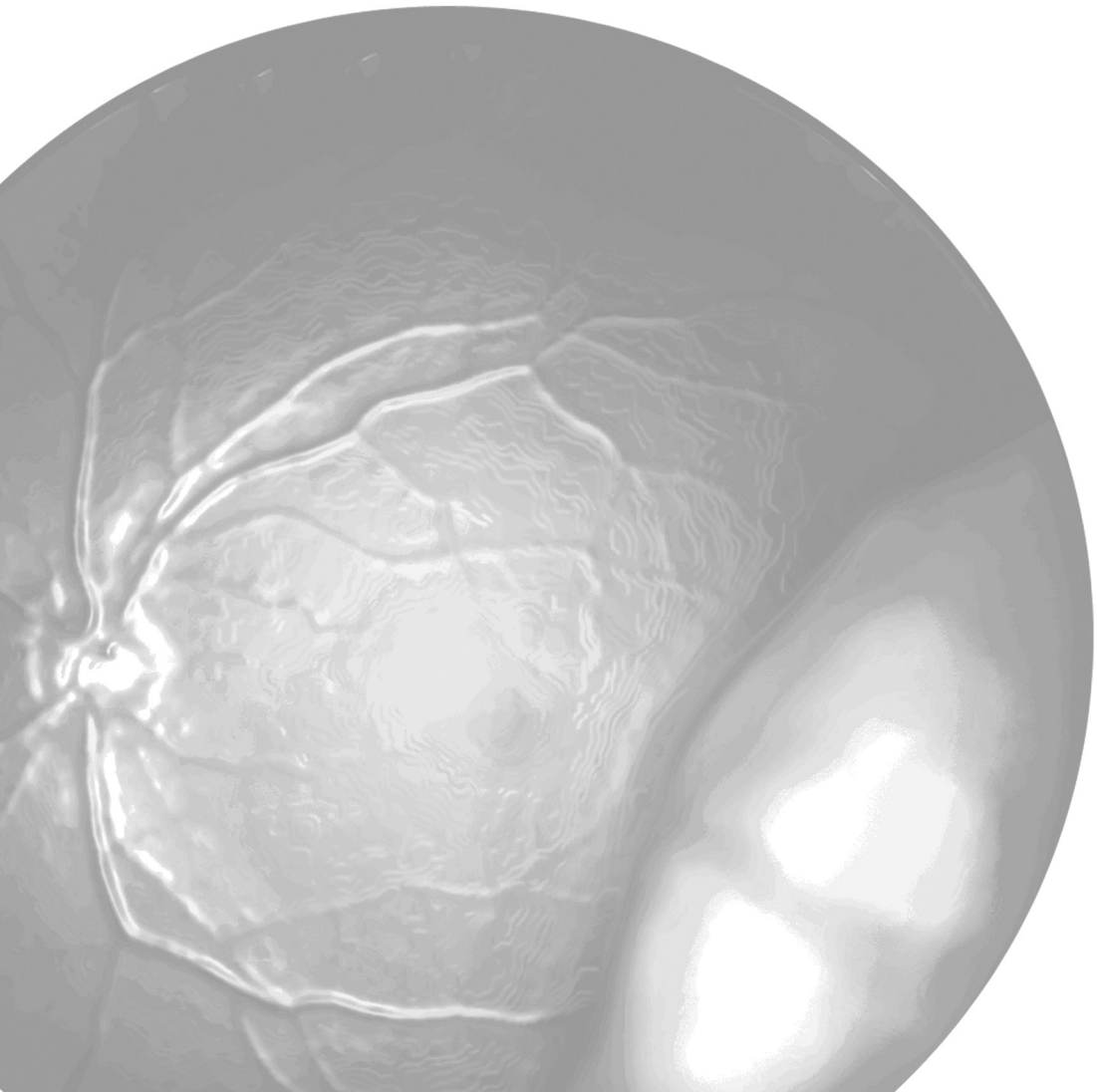
Chapter 1

Introduction



Aims of this thesis

This thesis focuses on two different topics: the first one involves the role of inflammatory processes in the eye with regard to uveal melanoma, and the consequences for patient prognosis. The contribution of macrophages to tumor growth and their capacity to modulate the immune system are studied. The second part of this thesis focuses on approaches to inhibit intraocular tumor growth by using a new strategy, i.e. combining Adoptive Cell Transfer, Long Peptide vaccination and monoclonal antibody treatment.
Understanding the inflammatory process and testing new treatments will help us to obtain a better treatment for primary uveal melanoma and its lethal metastases, thus improving patient survival.

Uveal melanoma

Uveal melanoma is the most frequent primary intraocular tumor in adults with an annual incidence of 6-8 cases per million people per year [1]. The location of the tumor in the uvea is approximately in 5% the iris, in 10% the ciliary body and in 85% the choroid [2,3]. Uveal melanoma may arise at any age, but it appears most frequently in the sixth decade of life [4]. A study in our own patients showed that the 5-year survival rate for patients with an enucleation is 72% and the 10-year survival is 59% [5]. Patients with uveal melanoma mainly die from liver metastases, but this tumor also disseminates to other sites such as the lung, bone and skin [6-8].

Treatment of uveal melanoma and metastasis

For a long time, enucleation was the only treatment for uveal melanoma. During the last 40 years, eye-saving treatments have been developed, such as local radiation by brachytherapy with different types of radioactive isotopes (Ruthenium-106, Strontium-90, Iodine-125 and Cobalt-60, Palladium-104) [9,10], sometimes in combination with Transpupillary Thermotherapy (TTT, so-called sandwich therapy) [11,12]. For this combined treatment, tumors are approached bilaterally: the apex is treated by TTT and the base of the tumor with a radioactive plaque.
Brachytherapy with radioactive plaques was shown to have the same effect on survival in medium-sized tumors as immediate enucleation [13]. Proton beam irradiation uses a very precise delivery system, treating large tumors and tumors located near the optic disc [14]. Stereotactic therapy is an alternative for proton beam therapy for large tumors [15]. Local resection is applied in some centres, for removal of small tumors, but the use of this technique depends largely on the experience of the surgeon [16-18]. Enucleation is still used for large tumors, for recurrences and when complications occur such as neovascular glaucoma.
All treatments help to control the primary tumor, but many have detrimental influences on the function of the eye, namely loss of sight by inducing cataract,

retinopathy, vitreous hemorrhage and neovascular glaucoma [19,20]. Furthermore, these treatments do not prevent the development of metastases from the primary tumor, since after introduction of these treatments, survival has not improved.
Metastases occur mostly in the liver (56-90%), lung, bone and skin. Treatment with systemic chemotherapy was shown to have limited success, since response rates are low; experimental treatment with a combination of chemo and immunotherapy (such as interferon, interleukine-2) provide slightly better results than monotherapy, but are also not the definitive solution for metastases, since survival is still poor [21]. Since the liver is the most frequent site of metastasis, some treatments focus on delivering therapy specific to this location. Intra-hepatic arterial liver perfusion, chemoembolization and resection of isolated liver metastases are being applied, and have been able to prolong survival in individual cases [22,23]. Despite sporadic successes, patients still do not have a median survival longer than 10-18 months after the identification of metastases [24].

Immunological features of uveal melanoma

Uveal melanomas are situated in a special location in the body, namely the immune-privileged eye. The immune system follows specific rules in this organ, which are shared with the brain and reproductive organs, probably because loss of organ function after an immune reaction will have detrimental consequences for the survival or reproduction of the host [25,26]. Tissues which are transplanted into these immune-privileged sites are not rejected, due to the deviant immunological laws. Furthermore, soluble antigens, which are introduced into the anterior chamber, spread to the rest of the body, inducing systemic tolerance, while usually an inflammatory response would occur, when the antigen is introduced elsewhere. This phenomenon has been described as Anterior Chamber Associated Immune Deviation (ACAID) [27,28]. One can imagine that antigens from tumors in the eye induce ACAID, leading to suppression of an immune response against an intraocular tumor. Therefore, these sites are creating an interesting niche for studying immunological processes, due to their deviant laws.
Another interesting phenomenon in intraocular tumors is related to HLA expression, which plays an important role in immune recognition. Often, HLA expression is an important prognostic factor for survival in patients with a tumor. In other malignancies, an increased HLA expression is often associated with a better survival, since this will enhance antigen presentation and as a result, more T cell activity to eradicate tumor cells [29-32]. Interestingly, Blom et al. [33], and later Dithmar et al. [34] and Ericsson et al. [35], reported that in uveal melanoma, a higher expression of HLA class I antigens was associated with a decreased survival. A possible explanation may be the hematogeneous spreading of uveal melanoma: when cells with a low HLA expression are present within the bloodstream, they escape from T cell-mediated lysis but are still discovered by NK cell immunosurveillance [36]. An increased expression of HLA Class II was also found to be associated with a worse survival [35].

Inflammation in uveal melanoma

Histopathological analysis of enucleated eyes shows the occurrence of inflammation in uveal melanoma. T lymphocytes, macrophages and blood vessels can be present in the tumor. Leukocytic infiltrates are found frequently in uveal melanomas. The lymphocyte population consists mainly of T cells, and several early studies identified the presence of lymphocytes in uveal melanomas as being a bad prognostic sign [37,38]. Uveal melanoma also contains populations of macrophages. De Waard-Siebinga et al. [39] analysed the presence of different types of infiltrating cells in uveal melanoma, and found CD3+, CD4+ and CD8+ lymphocytes, as well as CD11b+ and CD15+ cells, considered to be monocytes/ macrophages and granulocytes, respectively. CD15+ cells were rare, but CD11b+ cells were present in ~90% of tumors studied. A positive correlation was observed between the presence of CD3+ cells, CD11b+ cells and the level of HLA Class I expression.
Immune cells are recruited to the tumor, probably due to the fact that the tumor is recognized as hostile. According to the inflammation theory, leukocytes travel through blood vessels to either attack the tumor or restore homeostasis after damage by an immune reaction [40,41]. Previous studies have shown that a leukocytic infiltrate, high macrophage density and a high microvascular density are associated with a decreased survival in uveal melanoma, but why this occurs has not yet been elucidated [42,43].

Macrophages in uveal melanoma

Mäkitie and Kivelä [42] studied the relationship between the density of Tumor-Associated Macrophages (TAM) and patient survival in uveal melanoma. In 149 cases of choroidal or ciliary body melanoma enucleated between 1972 and 1981, CD68 was used as marker to determine the number of TAM. The tumors were divided into three groups: those with a few (17% of cases), moderate (51% of cases) or many macrophages (32% of cases). The number of TAM was associated with survival: the higher the density of TAM, the worse the survival. Higher numbers of TAM were also associated with female gender, large basal tumor dimensions, epithelioid cell type, heavy pigmentation, and a high microvascular density (MVD) in the areas with the densest vascularisation [43]. An association between the presence of a high number of TAM and a high MVD has also been observed in other cancers such as cutaneous melanoma [44] and breast cancer [45], suggesting a causal relationship.
The classical macrophage has been described as a key player in protecting the host from pathogens. Its main function is to phagocytose detrimental/hostile/ pathogenic cells and fulfilling its function as Antigen Presenting Cell (APC) to help other immunologically-active cells for guarding the host [46,47].
Recently, some new functions of these immune cells have been described and in the review on macrophages in Chapter 2, we describe these features and their implications and interpretations in uveal melanoma.

Mouse models

Several intraocular tumor models have been introduced to study uveal melanoma. Tumor cell lines of different origin have been inoculated into different compartments of the eye of various mouse strains. First, a distinction can be made between syngeneic or xenogeneic models. The most commonly used mouse strain in research is the C57BL/6 mouse. Tumor cell lines which are derived from C57BL/6 murine origin can be placed into the eye of this mouse, e.g. the B16 melanoma cell lines and the Ad5E1 cell line. These syngeneic models do not require immunological intervention to prevent rejection of these cell lines in the eye. Boonman et al has previously shown that due to the immuno-tolerant environment of the eye, it was possible to grow the highly immunogenic but syngeneic Ad5E1 cell line in the anterior chamber of a C57Bl/6 mouse [48].
For prevention of rejection of transplanted xenogeneic cell lines, the immune system of the host has to be downregulated. Normally, allo- or xenogeneic cell lines are rejected within 12 days in an immunocompetent host [49], but when immunomodulated or immunodeficient mice are used, these cell lines can grow in the murine eye. We have been able to transplant human cell lines into the eyes of immunodeficient mice, such as the Severe Combined Immuno Deficient (SCID) or nude mouse [50-52].
The place in which the tumor cells are inoculated makes a difference. In the first model, which was used for studying intraocular tumors, tumor cells were inoculated into the anterior chamber of the mouse [53]. An advantage of this model is that the tumor can be observed directly through the cornea without sacrificing the mouse. In another approach, tumor cells are injected intrachoroidally/subretinally, which corresponds to the human situation of uveal melanoma. Another way to create a uveal melanoma model comparable to the human situation, is by injecting tumor cells through the iris into the ciliary body, giving rise to ciliary body tumors. A disadvantage of intrachoroidal or subretinal tumor placement is, that it is difficult to follow tumor growth in time, since it is located at the posterior pole. Recent innovations are achieved by inserting a bioluminescent (such as luciferase) or autofluorescent gene sequence into the tumor cell line, creating the possibility to perform *in vivo* imaging, and to follow the process of tumor development with a 3D view, preventing the necessity to sacrifice many animals [54].

Immunotherapy

Uveal melanoma has been considered a tumor which is difficult to treat; several treatment options are being used, but successfully preserving sight as well as preventing metastasis formation seems to be utopia. Uveal melanoma remains a very aggressive tumor, which spreads systemically, leading to lethal metastases. One way to attack tumor cells systemically is to apply immunotherapy, which is based on triggering the natural surveillance system of our own body.
As mentioned before, several experimental treatments with immunotherapy have been applied to treat metastasis, thus improving survival. Interferon and

IL-2 are applied to stimulate the immune system, leading to aspecific responses against tumors. Unfortunately, many complications occur with this method of immunotherapy, making these aspecific approaches of immunotherapy less desirable [21].
The combination of chemo- and immunotherapy has achieved some success [55,56]. Such combination therapies also involve a lot of side effects, due to their aspecific features.
Therefore, we wanted to apply new immunotherapeutic approaches to create specific immune responses resulting in enhancement of tumor cell eradication, while avoiding side effects. As a consequence, several components of the immune system have been selected as new treatment options. One of the components used are cytotoxic T lymphocytes (CTL, the CD8+ T cell). This cell is directed against hostile cells [57]. The main goal of different study groups in the world is to activate these CD8+ CTLs in order to eradicate melanomas or other malignancies. Studies have been performed on the use of endogenous T cells recognizing so-called Tumor-Associated Antigens (TAA), which are expressed on tumor cells, leading to effective killing of the pathogenic cell [58-60]. It is difficult to find antigens which are only expressed on tumor cells and not on healthy cells. Often, self-antigens that are expressed on normal as well as on tumor tissue are used as targets, which can lead to autoimmune pathology. Another major problem with this approach is that endogenous T cells may be tolerant against these self-antigens, and that immune responses are of low quality [61,62].
Several strategies have been introduced for refinement of CD8+ CTL therapy. To circumvent self-tolerance, ex vivo activation of auto-reactive T cells for specific TAA has been introduced as a concept for more effective tumor eradication [63]. Tumor-infiltrating lymphocytes (TIL) isolated from melanoma patients have been activated ex vivo and re-infused into patients for treating metastases, with some promising results: this Adoptive Cell Transfer (ACT) of auto-reactive T cells for metastatic melanoma resulted in a 49-72% objective response rate according to studies by Rosenberg et al. [64,65]. Furthermore, studies in which leukemic diseases are treated with alloreactive Donor Lymphocyte Infusions (DLI) after a bone marrow transplantation, showed effective eradication of malignant cells in the peripheral blood [66]. Additionally, ex vivo modification of T cell receptors with TCR gene therapy led to better recognition and antigen processing by creating highly avid T cells for the TAA epitopes, and gave better results in tumor targeting [67,68].
Although ACT showed some promising results, the major problem is maintaining the T cells' in vivo function, i.e. by keeping high frequencies of activated transferred T cells. The peripheral tolerance of tumor tissue and the host immune system will lead to clonal deletion or anergy of T cells, while suppressive mechanisms of regulatory T cells (Tregs) also lead to less activity of adoptively-transferred cells [69,70]. Use of additive vaccines, such as influenza virus, DNA vaccination or short peptides did not improve the persistence of T cells [71-73]. In order to circumvent these problems, host conditioning by Total Body Irradiation (TBI) or chemo-ablation with cytostatic agents has been shown to lead to maintenance of high frequencies of autoreactive T cells against the tumor [65,70,71,74,75]. Although these measurements improve T cell efficacy, the

immunodeficiency by ablation of the host immune system resulted in many side effects, allowing opportunistic infections and other complications. The main challenge for ACT will be expanding and maintaining functional transferred T cells, without host conditioning.

Another major improvement in immunotherapy has been the development of improved vaccines against tumors. Patients are often vaccinated with minimal short peptides (non-modified tumor/self peptide antigens), which have a low affinity for the T cell receptor, leading to ineffective anti-tumor responses. Several murine studies showed that combining these self peptide antigens with viral infection or vaccinia helped to boost the natural immune system [76,77]. Recently, Kenter and Melief et al published that the use of long peptides (Synthetic Long Peptides, SLP) gave better anti-tumor responses in premalignant lesions, namely Vulvar Intraepithelial Neoplasia (VIN) caused by Human Papilloma Viruses. Using long peptides resulted in dendritic cell-focussed processing leading to optimal CD8+ T cell activation and the use of sequences that coded for helper epitopes led to additional recruitment of helper T cells [78-80]. The functionality of SLP has to be tested in other malignancies.

The additional use of adjuvants, such as CpG, Toll-like receptor-9 ligand (TLR9L), has been demonstrated to be effective against several cancers, but also in inflammatory diseases, such as hepatitis. Aldara (Imiquimod), Toll like receptor-7 ligand (TLR7L), is extensively used in dermatology to treat benign, but also (pre)malignant diseases. This ligand enhances antigen presentation and activation of the innate immune system such as dendritic cells, leading to effective eradication of skin lesions. Therefore, Toll like-receptor ligands could enhance activation and clonal expansion of CTL by improving antigen presentation [81,82]. Adding this component to immunotherapy should hypothetically lead to a more effective tumor eradication.

Use of monoclonal antibody against tumors

Monoclonal antibodies have extensively been studied for their efficacy to treat tumors. The working mechanism is based on the fact that some have a direct apoptotic effect on cells by triggering receptors on cancer cells [83]. Other mechanisms are recruiting cytotoxic cells, such as macrophages, which is known as antibody-dependent cell mediated cytotoxicity (ADCC) [84] or by activating the complement system, leading to direct cell toxicity, known as complement-dependent cytotoxicity (CDC) [85]. An alternative approach is to conjugate the monoclonal antibody to a toxin, a cytotoxic agent, a radioisotope or a chemotherapeutic agent [86].
Many studies report on the use of monoclonal antibodies, but did not show effective anti-tumor efficacy. Recently, some monoclonal antibodies, such as Panorex (anti-17-1A for colon cancer), Herceptin (anti-HER2 for breast cancer) and Rituxan (anti-CD20; rituximab; IDEC-C2B8 for leukemia and non-Hodgkin's lymphomas) either in combination with or without chemotherapy, have been demonstrated to be effective in hematologic malignancies and solid

tumors [87-90].
There are several obstacles to successful therapy with monoclonal antibodies. Tumors could be quite heterogeneous, so the antigen, which the monoclonal antibodies targets, is often not expressed by all tumor cells. Furthermore, the blood vessel supply to the tumor is not always optimal, due to ischemia [91], so if monoclonal antibodies need to be delivered hematogeneously, it may be difficult to bring them to the tumor site. Another problem with monoclonal antibodies is that they are often generated by murine cells, leading to possible immune responses in humans against these antibodies [92]. Such immune responses not only decrease the efficacy of monoclonal antibody therapy, but also eliminate the possibility of multiple treatment rounds. Therefore, humanized monoclonal antibodies have been developed to circumvent these responses.

Outline of this thesis

In this thesis, I focus on the inflammatory process that is present in the eye with uveal melanoma and analyse how the immune system can be modulated to target the tumors in order to find an effective therapy for this malignancy. Several previous studies have already shown that immunological infiltrates are present in uveal melanoma, and show an association with prognosis. One of the infiltrating cells is the macrophage. A higher density of these cells is associated with a decreased survival in uveal melanoma. Therefore, we reviewed (Chapter 2) existing information on the role and function of this immune cell and translated this for eye diseases and especially for uveal melanoma. In Chapter 3, we describe that different infaust prognostic markers in uveal melanoma occur together with monosomy 3, a genetic aberration which is associated with a decreased survival. As mentioned above, infaust prognostic markers are immunological parameters, which represent together a state of inflammation. As mentioned before, macrophages play a key role in tumor growth. Mantovani [93] introduced the so-called M1 and M2 paradigm, in which subtypes of macrophages are identified by their function. M1 macrophages are immuno-stimulatory, anti-angiogenic and tumor-suppressive, while M2 macrophages are more pro-angiogenic and tumor-promoting. Since as far as we know, nobody has studied the presence of tumor-promoting M2 macrophages in uveal melanoma, we determined these cells (in Chapter 4) with immunofluorescence in a set of 50 patients.
Since we know that an inflammatory phenotype often exists in an eye containing a uveal melanoma, we hypothesized that the infiltrate is attracted by a local production of cytokines. We therefore analysed the presence of inflammatory cytokines (Chapter 5) in the aqueous humor from uveal melanoma-containing eyes and found that many were highly expressed.
We also wondered whether these cytokines were related to the presence of macrophages and especially the tumor-promoting M2 macrophages.
Blood vessels play an essential role in tumor growth and maintenance. These structures are essential to supply nutrients to the tumor and they are pathways for the tumor to metastasize. Angiogenesis occurs under certain circumstances,

such as hypoxia, but also due to inflammation. The latter process triggers blood vessel growth, partly mediated by macrophages. We determined (Chapter 6) whether ingrowth of tumor cells in blood vessels of the eye was associated with other prognostic markers and survival.
Furthermore, we wondered about the relationship between macrophages and tumor growth. In Chapter 7, we describe the role of macrophages in intraocular tumor growth in a mouse model. The syngeneic and poorly immunogenic B16F10 cell line was placed into the anterior chamber of a C57BL/6 mouse, in the same location as in the previous models of Boonman en Schurmans from the same laboratory [48,94]. We modulated the presence of macrophages with subconjunctival administration of a drug, clodronate liposomes, and determined the intraocular growth in both young (6 weeks) as well as old (10 months) mice. We know from the M1 and M2 macrophage paradigm, that the different phenotypes could modulate tumor growth. The rationale for studying age, is that several studies from Apte, Kelly, and Espinosa-Heidmann [95-97] demonstrated that a different macrophage polarization is present in mice of different ages. In young mice, macrophages are polarized towards an M1 phenotype, in old mice towards an M2 phenotype.
In Chapters 8 and 9, we applied immunotherapy as experimental treatment of murine tumors, as a model for uveal melanoma. In Chapter 8, we studied immunotherapy with the use of gp100-based (one of the melanocyte differentiation antigens) adoptive cell transfer (ACT) of T cells in combination with long peptide vaccination. Previous studies described that in order to achieve high frequencies of T cells after ACT, one needs host conditioning, which is known to be accompanied by many complications. Furthermore, long peptides have an effect in premalignant lesions in the vulva, but have not been tested in melanoma. Therefore, we combined ACT together with the long peptides, in order to eradicate tumors effectively. Since we know that B16 melanoma in C57BL/6 mice has been described as a very aggressive type of tumor due to its poor immunogenicity and tumor evasive mechanisms, we tested this newly combined treatment in this syngeneic tumor model.
Since we already know that therapeutic long peptide vaccination alone is not effective in a mouse melanoma model, we describe in Chapter 9 the effect of the additional use of a monoclonal antibody (TA99) which is directed against Tyrosinase Related Protein-1 (TRP-1) antigen. Injection of both of these two different components should work synergistically.
The implications of these studies regarding the inflammation status in uveal melanoma and immune modulation in experimental models for intraocular tumors are discussed in the final chapters of this thesis.

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