Inflammation and immunomodulation in uveal melanoma

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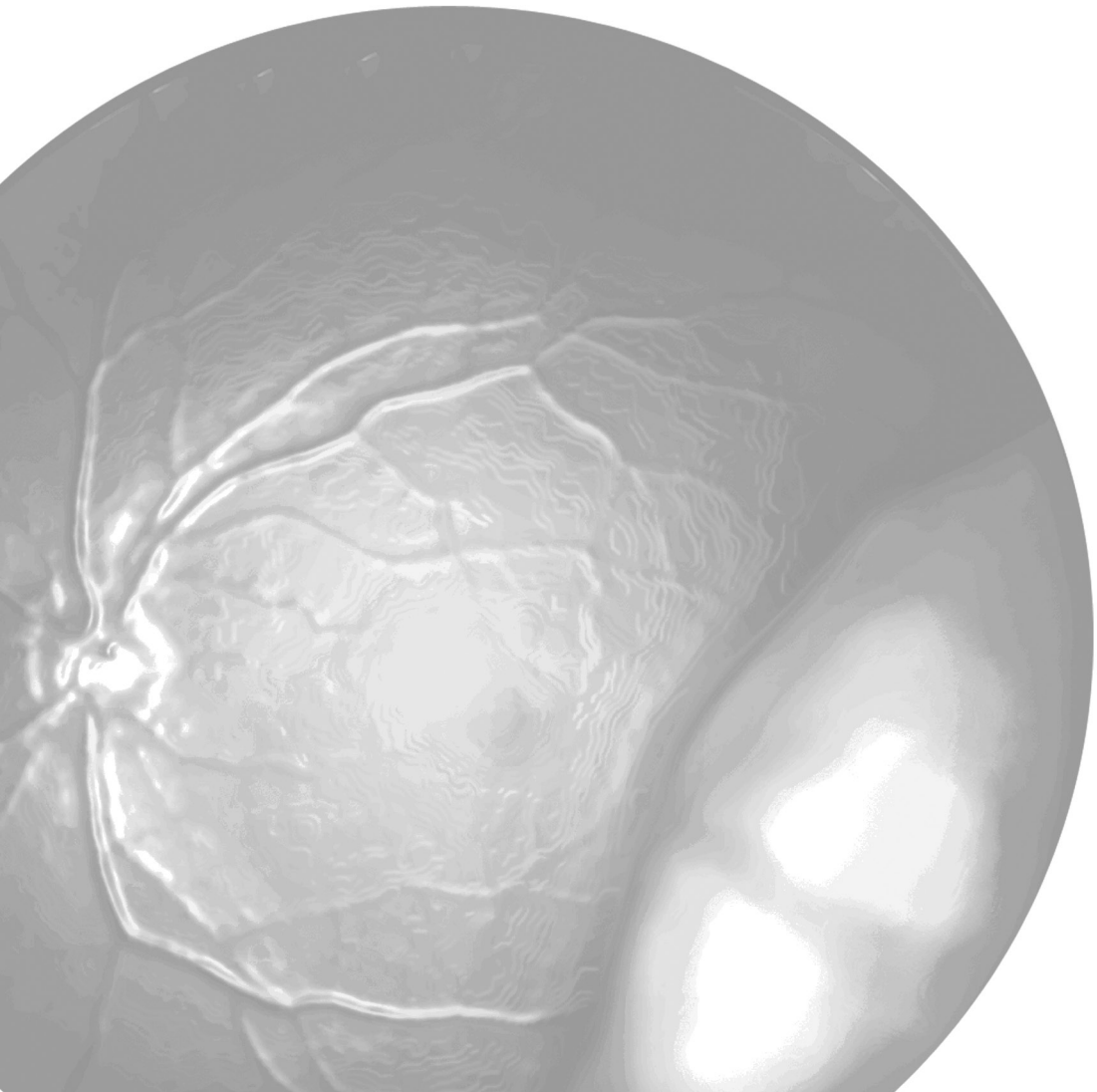
Chapter 10

Synopsis, conclusions and future perspectives



Synopsis

While the treatment of intraocular uveal melanoma is usually effective, survival is still poor, since micrometastases are already present at diagnosis of the primary uveal melanoma and established metastases of this tumor are difficult to treat[1]; although some further insight in the etiology and pathogenesis of this lethal type of cancer has recently been obtained, a lot of questions remain on how this tumor develops and how we can prevent progression.
In this thesis, one major aspect of uveal melanoma was studied, namely inflammation and its modulation. In general, the immune system plays a role in tumor development. This is not different in uveal melanoma: in this thesis we show that inflammation predicts a worse prognosis. An active state of inflammation is seen in uveal melanoma-bearing eyes, showing the presence of macrophages, lymphocytes and a high expression of HLA Class I and II[2-5].
Focusing on macrophages (Chapter 2), from which we know that high densities intratumorally are associated with a decreased survival, we reviewed the role of these cells in the immune system and translated this into ophthalmology and especially in the settings of uveal melanoma. The macrophage is described to be multifaceted in uveal melanoma: in different environments and different animal models for intraocular melanoma, this cell has a tumor-promoting or tumor-suppressing role. Its function depends on what kind of phenotype the macrophage has, such as Antigen-Presenting Cell, M1 (tumor-suppressive), M2 (tumor-promoting) or Myeloid Derived Suppressor Cell (tumor-promoting). Finding ways to modify macrophage differentiation into a desired phenotype will create therapeutic possibilities, and will lead to more chances to find an effective method for tumor eradication.
In Chapter 3, we described the inflammation state in uveal melanoma, which is associated with a decreased survival, as the so-called "inflammatory phenotype" and found that this was associated with another well-defined marker for bad prognosis in uveal melanoma, namely monosomy 3. Apparently these genetic and immunological features of uveal melanoma are linked to each other. This was the first time, that these two phenomena were shown to be related, and apparently genes located on chromosome 3 may determine the immune status in the tumor, but the exact genes causing this phenomenon have not been identified.
Furthermore, we highlighted one of the components of the inflammatory phenotype in Chapter 4, namely the macrophage. According to Mantovani's M1 and M2 macrophage paradigm, two types of macrophages exist, with different functions[6,7]. The M2 macrophage is described to be tumor-promoting, partially due to its capacity of matrix remodelling, its pro-angiogenic feature and immuno-suppressive capacity. A uveal melanoma shows an abundance of M2 type macrophages and the density of these cells correlates with monosomy 3 and decreased survival, indicating that loss of one copy of chromosome 3 leads to more macrophage infiltration and immune stimulation. A possible explanation can be that the genes, which are coding for inhibition of macrophage recruitment, are located on this chromosome 3, and loss of those

specific genes will lead to massive infiltration.
Since we described that an inflammatory phenotype can exist in uveal melanoma, one of the main questions is how this can easily be determined in an eye with a tumor. Nowadays, Fine Needle Aspiration Biopsy (FNAB) is used to puncture the tumor intraocularly to obtain samples for chromosome 3 analysis, but this method is invasive. Therefore (Chapter 5) we wondered whether we could instead take a sample of aqueous humor, which is relatively easy. Chemokines and cytokines determine the attraction of immune cells, and our hypothesis is that the presence of these proteins is associated with an inflammatory phenotype. We performed a Multiplex Cytokine array on different samples taken from enucleated eyes, and while we find that aqueous humor of uveal melanoma patients contains significantly more cytokines and chemokines than in healthy patients, the cytokines were not associated with other prognostics markers and do not predict survival. Cytokines and chemokines are produced in the eye in many other inflammatory ophthalmic diseases, including uveitis and acute retinal necrosis [8], not only by immune cells, but also by retinal cells and in our case, tumor cells. We now think that cytokines are a reaction to the tumor, and can be a possible target for therapy. Several studies have been performed on antibodies against cytokines and have been shown to be effective in inhibition of inflammatory cells and on downstream targets[9]. Experimental studies can take place using such antibodies in an animal model.
In Chapter 6, we analyzed the role of tumor cell invasion into blood vessels in human uveal melanoma, and observed that this was associated with a worse prognosis; one can image that macrophages aid in this process, since remodelling of the extracellular matrix is one of the functions of these cells, and this will lead to invasion of tumor cells into this structure. This feature is described to be a characteristic of tumor-promoting M2 macrophages. For further confirmation of this reasoning, we should compare the presence of M2 macrophages with the results of tumor cell invasion in blood vessels.

On the one hand, we demonstrated the presence of many different adverse aspects of the inflammatory process in uveal melanoma, but on the other hand, we want to use the immune system for our main goal: to find a treatment to eradicate this tumor, using its immunological features. In Chapter 7, we found that macrophages and age play an important role in the tumorigenesis of a syngeneic tumor in an immunocompetent mouse. We demonstrate in this chapter that when the eyes of old mice are depleted of macrophages, this prevents tumor growth, while in young mice, macrophage modulation induced hardly any difference. With immunohistochemistry and gene expression analysis, we showed that the observed effect in old mice was due to the depletion of tumor-promoting, pro-angiogenic and immuno-suppressive M2 macrophages. Interestingly, this phenotype of cells is constitutively present in high amounts in eyes of aged individuals. The age-related change of the immune cell (i.e. the macrophage) phenotype determines the tumorigenicity of a tumor in a mouse and is apparently a physiologic aging phenomenon. This finding is also relevant for uveal melanoma patients, since we know that

this tumor develops in relatively older people, and it has also been found that higher numbers of tumor-infiltrating macrophages are associated with a worse prognosis.
In Chapter 8, we used adoptive cell transfer (ACT) of transgenic T cells specific for gp100 (pmel cells) and 20-mer long peptide vaccination as prophylactic treatment for intraocular melanoma. Gp100 is one of the melanoma-differentiation antigens and is expressed on pigment and melanoma cells. Activation of the adoptively-transferred T cells was quite effective, since we managed to maintain high and effective T cell frequencies systemically in the mouse without host conditioning. However, multiple vaccinations with the 20-mer peptide led to a massive cytokine release (cytokine storm), with a Systemic Inflammatory Response Syndrome (SIRS) and multi-organ failure as result, as can also be observed with Graft-versus-Host-Disease or studies using the monoclonal antibodies anti-CD28 or anti-CD40 [10,11]. Since ACT has already been used in clinical studies, this study demonstrates that we have to be careful when using this combination treatment. Prevention of these lethal symptoms can probably be achieved by using a lower affinity peptide or mineral oil as solvent (Freud's adjuvant (IFA)/Montanide instead of Phosphate Buffered Saline (PBS)), which will result in slow release and presentation of the peptide to the antigen presenting cells (APC). Ongoing studies analyse the efficacy and anti-tumor effect of our pmel cells after treatment with short peptides or montanide as solvent.
In Chapter 9, we demonstrate the effect of a monoclonal antibody (TA99), which acts on TRP-1, another antigen expressed on melanoma[12,13]. We show that a 2 times-administration regimen in combination with therapeutical long peptide vaccination leads to a significant delay of subcutaneous outgrowth of melanoma. Our idea of the working mechanism of TA99 is that it does not enhance T cell priming, since we have proven that combination treatment of TA99 and vaccination does not lead to a greater expansion of endogenous T cells. Therefore, our hypothesis is that TA99 modulates the tumor micro-environment, by changing macrophages, NK cells and B cells, since TA99 is known to act on Fc-gamma receptors[12] and will lead to inhibition of tumor-suppressive cells and thus to more effective tumor cell eradication.

Considerations regarding animal studies

In Chapter 7, we have already mentioned that changing of the macrophage phenotype is apparently a physiological aging phenomenon, and can determine the tumorigenicity of a tumor in a mouse. This concept gives an additional insight to the most common ideas regarding tumorigenesis, which describe cancer to be mainly associated with oncogenes and tumor suppressor genes. The general paradigm is, that tumors arise when different genetic mutations occur, and these accumulated defects over time will lead to loss of growth inhibition of mutated cancer cells[14,15]. We now describe that immune cells sculpture the tumor tissue environment contributing to oncogenesis and

introduce this as a new idea in uveal melanoma.
Furthermore, our finding that tumors grow differently in older animals has also implications; since most animal studies are performed in young individuals, this does not represent the situation in humans, in which cancer is an age-related disease. Therefore, results in studies with young animals have to be interpreted with care.

Furthermore, old mice carry many M2 macrophages, and at the same time, angiogenic and immunosuppressive markers are expressed. Further studies can be performed to see whether there are more blood vessels present in tumors in old mice, since we associated the M2 macrophage with a pro-angiogenic function. However, this can be a problem, since we are dealing with a small tumor in the anterior chamber of a mouse, which comprises only 4 mm^3. We know that tumors which are 2 mm large in diameter can still grow without blood vessels. Still, angiogenic signals can be turned on, and no visible changes in vasculature can be observed. Furthermore, one can ask whether the M2 macrophages are supporting tumor growth by acting as Myeloid Derived Suppressor Cells, inhibiting cytotoxic T cells and other tumorilytic cells. Additional functional experiments with knock-out mice are indicated and we can analyse T cell infiltration intratumorally to observe the effect of immunosuppression of M2 macrophages.
It seems that M2 macrophages support tumor growth, and thus this polarized version of the macrophage is causing a problem. Simply stated, if we manage to convert the M2 macrophage towards an M1 macrophage, this could prevent the detrimental effect of the M2 macrophage on tumor growth[16,17]. Although this sounds simple, this idea has some drawbacks: 1) we know that the M1/M2 distribution in a mouse is a relative, and not an absolute polarization. After changing the balance, how far can we go in order to prevent disturbance of the homeostasis? 2) Furthermore, we do not have specific signals to change fully differentiated M2 macrophages back to the precursor stage and then stimulate them specifically into an M1-type. One can imagine applying bonemarrow irradiation in old mice to deplete all M2 macrophages and then stimulate the new bone-marrow-produced monocytes specifically into M1-type macrophages. What kind of effect will this rigorous measurement have on the host? Also, lymphodepletion can provide a chance to opportunistic infections, while modulation towards only an M1 phenotype, will leave the host without M2 macrophages. The latter subtype is essential for tissue repair and remodelling, and without these cells this could have detrimental effects as well.

Our studies for intraocular melanoma are performed with the poorly immunogenic B16 cell line in C57Bl/6 mice. This cell line grows progressively in the anterior chamber, without rejection. Previous studies performed in our and Niederkorn's lab used the highly immunogenic Ad5E1 cell line, which grows maximally for 12 days, and could then be either pristinely or phtysically rejected in the eye[18-20]. In case of pristine tumor rejection, the eye structures remain intact, while phtysical rejection of the tumor leads to a simultaneous destruction of the whole eye.

Boonman[19] demonstrated that macrophages played a key role in this process of rejection, since depleting these cells with subconjuncitval injections of clondronate liposomes, showed no pristine rejection and long time maintenance of these cells in the Anterior Chamber. Furthermore, CD4+ T cells and IFN-γ are essential in rejection of the tumor without collateral damage to the ocular tissue[20].
Recently, Niederkorn's [21] lab showed that phthysical rejection of the Ad5E1 cell line is predominantly M1 macrophage mediated. iNOS, known to be quite characteristic for M1 macrophage functionality, was essential in this process and is responsible for the collateral damage to the other ocular structures, leading to phthysis.
The studies with the Ad5E1 cell line were essential to demonstrate the process of tumor rejection, which will give us more insight how to get rid of a tumor and how macrophages might contribute to tumor rejection. However, this model is less representative than using a poor immunogenic, syngeneic cell line, like B16, which will not be rejected, as usually observed with human tumors. Therefore we conducted our research using B16 in C57BL/6 mice for studying the contribution of macrophages in the tumorigenic process. Since this tumor is poorly immunogenic and not rejected, we can exclusively study the tumor-supportive role of macrophages. Thus, the role of macrophages in the Ad5E1 cell line is predominantly tumorlytic, while in the study with B16 melanoma they have a tumor-supportive function. A possible explanation for the fact that these cell lines trigger different responses of the macrophages in the same mouse could be caused by the fact that the Ad5 mouse embryo cells (AD5MEC) are created in vitro, while the B16 is a cultured cell line from a spontaneous murine melanoma and possesses immune-modulating characteristics similar to human tumors.

In our vaccination studies, we described that long peptides in combination with ACT of specific T cells will lead to a SIRS, but what is a long peptide length? Short peptides consist of 9 amino acids, but long peptides exist in 20-mer up to 34-mer. In our studies with vaccination, we used 20-mer peptides, while studies which describe that long peptides are effective against premalignant lesions of the vulva (VIN lesions) use the patented Synthetic Long Peptides (SLP) which are at least 25-mer peptides[22,23]. Van der Burg and Melief[24,25] have already described that the spreading of 9-mer short peptides through the whole body is effective, while 34-mer peptides remain at the injection site and do not travel further than the draining lymph node. In our study, the 20-mer peptides remained local, but were capable of inducing SIRS. We have to perform further research on how spreading of peptides is related to activation of T cells and inducing a cytokine storm. Another relevant remaining question is whether the 34-mer will cause the same symptoms, but according to previous studies of Van der Burg en Melief[24,25], spreading of this peptide is not effective systemically, hypothetically thus not inducing systemic activation of specific T cells and thus not leading to SIRS as a consequence.

Conclusions and future perspectives

In this thesis we stress that an inflammation state can be present in uveal melanoma and that this is associated with a bad prognosis. Since this is an important factor, modulation of the immune system could give possible therapeutic options. It has already been described for a decade that intratumoral macrophages are associated with decreased survival of uveal melanoma patients, but no sufficient explanation has been found. We show that modulation of this cell leads to tumor growth inhibition. Furthermore, the combined use of ACT and peptide vaccination provides enhanced targeting of tumor cells, leading to massive destruction of cancer. Although this seems promising, only targeting the tumor cells themselves is not sufficient, it is essential to also focus on cells which have a supportive role in the tumorigenic process[26].

We know that some monoclonal antibodies, such as TA99 used in our study, target tumor cells specifically, but can also modulate immune cells in sculpturing the tumor tissue environment.

New therapies in the field of oncology are the application of tyrosine kinase and angiogenesis inhibitors[27,28], showing effective possibilities against tumor growth. Research projects worldwide show that these drugs also modulate components of the immune system, and especially the microenvironment. Preliminary results from our laboratory show that these tyrosine kinase inhibitors can modulate macrophages. Angiogenesis inhibitors create possibilities for influencing angiogenesis and pro-angiogenic cells. Therefore, an interesting option is the use of a combination of these drugs, modulating the tumor micro-environment with vaccination focusing specifically on tumor cells, since synergistic mechanisms are triggered and lead to enhancement of anti-tumor effectivity.

This thesis shows that a synergistic approach of a combination of tumor-cell targeting and modulation of the micro-environment is a prerequisite for effective treatment of cancer.

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