Regulation of inflammation in uveal melanoma

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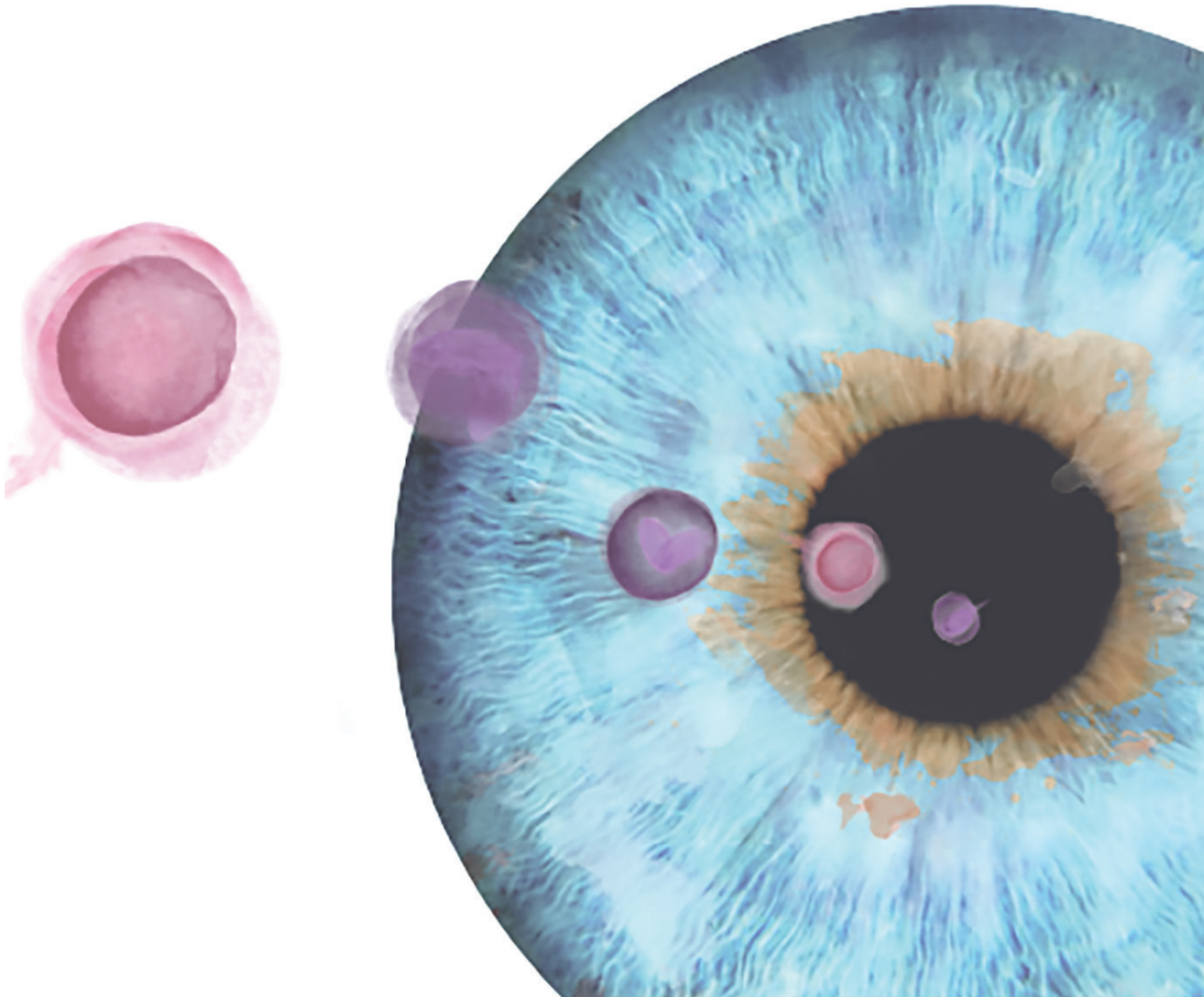
Chapter 8

Summary and discussion



Summary and discussion

While Uveal Melanoma (UM) is quite rare, it is often a deadly disease, with 50% of UM patients developing metastases (Augsburger 2008). The increased risk of metastases is associated with loss of one chromosome 3 and a mutation in the BAP1 gene in the tumour cells (Harbour 2010), while other genetic factors such as additional copies of chromosome 8q or mutations in the SF3B1 gene also play a role (Versluis 2015, Yavuzyigitoglu 2016). Loss of one chromosome 3/BAP1 expression in UM is associated with the presence of an inflammatory phenotype, although the mechanism that leads to inflammation has not yet been properly identified (Robertson 2017, Gezgin 2017). The survival rate of UM has not improved over the last five decades (Roelofsen 2021), and even the introduction of the new immune checkpoint inhibitors has not led to improved survival of UM patients (Rodrigues 2019).

The purpose of this thesis is to increase insight into the regulation of inflammation in UM. We study the main features of the inflammatory phenotype in UM (the presence of tumour-infiltrating lymphocytes and macrophages and the expression of HLA antigens), analysing the relation of these factors with the NFkB system, epigenetic HDAC regulators and miRNAs, and finally the relation with the LAG3 immune checkpoint molecule.

A comprehensive overview regarding the HLA system in UM showed that the level of HLA expression is determined by tumour genetics (**chapter 2**). HLA molecules are cell surface glycoproteins that present antigens to the immune system. This molecular mechanism can be hijacked by tumour cells and expression of HLA molecules downregulated to avoid T cell recognition. While specific HLA antigens may be downregulated, the overall expression of HLA antigens in UM may be upregulated: a high expression of HLA Class I and II is associated with high-risk tumour characteristics (de Waard-Siebinga 1996, Blom 1997, Ericsson 2001).

As already shown by Maat in 2008, upregulation of backbone HLA Class I is seen in tumours with loss of chromosome 3 (Maat 2008). However, UM cells might alter the expression of specific alleles or loci, which helps to avoid T cell immunity. This could take place at different levels such as genetic haplotype loss, individual allele loss and complete HLA-A or HLA-B loss (Hurks 2000, Anastassiou 2003). When investigating the expression of individual HLA-A and HLA-B alleles in

UM cell lines, we found defects in HLA-B locus which could not be revived by IFNɤ, although a genetic analysis showed the presence of the specific genes. This confirmed earlier results (de Waard-Siebinga 1995, Hurks 2000).

Following our review of the published literature about HLA expression as an inducer of metastasis in UM, we next investigated the possible role of the NFkB pathway in the inflammation of high-risk UM (**chapter 3**). NFkB is a transcription factor activated by inflammatory cytokines which, upon activation, is known to enhance the expression of proliferative, anti-apoptotic and pro-inflammatory downstream genes, affecting cancer cells at an early stage (as reviewed by Taniguchi 2018).

Using data from an Illumina mRNA array and long-term follow-up, we analyzed the mRNA expression of inflammatory markers in 64 primary UM samples. We found that components of the NFkB pathway are positively associated with the expression of HLA-A, HLA-B, major HLA Class I regulators including IRF1 and IRF8, and components of the antigen-processing machinery TAP1 and TAP2. Moreover, we highlighted the fact that the NFkB pathway is associated with infiltrating inflammatory cells, and with the tumour's chromosome/mutation status: high levels of HLA-A, HLA-B, NFkB1 and NFkB2 expression were associated with loss of one chromosome 3 and loss of expression of BAP1, which characterizes high-risk tumours. It may be that upon chromosome 3 loss combined with a BAP1 mutation, the inflammatory pathway loses its normal regulation. It is important to consider the possible role of PPAR-ɤ in NFkB regulation: this gene is located on chromosome 3 and may therefore be influenced by chromosome 3 loss. The reason for the high expression of NFkB pathway components in M3 tumours might be explained by insufficient negative regulation provided by PPAR-ɤ.

Inflammatory cytokines such as TNF-α, IL-1 and IL-17 can upregulate NFkB in colon cancer (Terzic 2010); here in UM, similarly, the inflammatory cytokines might contribute to the upregulation of this signalling pathway in the tumour cell which in turn can lead to the upregulation of HLA Class I and secretion of chemoattractants, thereby further enhancing local inflammation.

In addition to genetic aberrations, epigenetic changes may also alter gene expression. Identifying epigenetic alterations in high-risk UM is very important as the effects could be reversed by certain

drugs, and specific changes have been associated with the risk of metastases development (Robertson 2017). Different types of epigenetic regulation have been observed in the development of different cancers such as cutaneous melanoma, with DNA methyltransferases and Histone deacetylases playing a major role (Sigalotti 2010).

In **chapter 4**, we analysed the distribution of a wide range of histone deacetylase (HDAC) enzymes and a histone methyltransferase, enhancer of zeste homolog 2 (EZH2), and compared the level of their expression between low and high-risk UM tumours (disomy 3 = D3 vs monosomy 3 = M3 tumours). Some HDACs (HDAC1, HDAC3, HDAC4, and HDAC8) were higher while HDAC11 was lower in M3 compared to D3 tumours. The high expression of HDACs in high-risk UM tumours could be a possible mechanism for the enhancement of inflammation since HDAC enzymes have been shown to play a stimulating role in NFkB and IFNɣ signalling (Shakespear 2011). The association between a low expression of HDAC11 and M3 can be explained by the location of the HDAC11 gene on chromosome 3.

Because we found that HDACs are expressed in UM and are co-expressed with HLA Class I, we focused on the potential use of a combination of immunotherapy and anti-HDAC chemotherapy. To take an initial step towards this idea, we performed in-vitro studies using the HDAC inhibitor Quisinostat, which reduced cell growth in all three tested UM cell lines (Heijkants 2017). Although Quisinostat reduced UM cell growth, it also modified the expression of HLA Class I gene expression, increasing it both at the protein and mRNA level. The upregulation of HLA Class I is possibly due to the drugs ability to open the chromatin structure by inhibiting de-acetylation and increasing gene expression. This could have consequences: if the metastasis has already developed and reached the liver, the use of this drug could enhance HLA Class I expression and thereby T cell-mediated lysis; if only the primary tumour is present, this drug might contribute to the progression of the disease by further upregulation of HLA Class I, which may block NK cell-mediated lysis in the blood.

As we observed that especially high-risk UM showed a high expression of different epigenetic HDAC enzymes, together with upregulation of HLA Class I, we wondered whether HDAC expression is also associated with the presence of infiltrating leukocytes. Indeed, the expression of some of the HDACs (1, 3 and 8) was related to the presence of lymphocytes (**chapter 5**).

These HDACs were subsequently identified as being susceptible to upregulation by IFNɣ. This also explains the positive correlation observed between several HDACs and the loss of one copy of chromosome 3: it is likely that not only HLA expression but also expression of these HDACs is stimulated by the secretion of inflammatory cytokines.

Not only HDACs are involved in epigenetic regulation, but there are many other regulators as well. In **chapter 6** we focused on a set of non-translational RNAs named miRNAs as epigenetic regulators. MiRNAs have been shown to be involved in the progression of UM (reviewed by Smit 2019). We looked at the distribution of 125 miRNAs for which we had Illumina array expression information from 64 UM and investigated their association with different factors related to the inflammatory characteristics in UM. Since we know from previous studies that elevated HLA Class I expression is related to a bad prognosis and chromosome 3 loss, we wondered whether we could find any associations between miRNA expression and HLA-A and HLA-B expression levels. We found that among the 125 miRNAs, 24 showed significant associations: four miRNAs (22, 155, 635, and 1276) were positively correlated with HLA-A and HLA-B expression, while the other 21 miRNAs showed a negative correlation. Moreover, miRNAs with a positive association with HLA expression were often upregulated in M3 UM, while the opposite pattern existed for many of the downregulated miRNAs.

Loss of chromosome 3 together with a mutation in the BAP1 gene probably affects the normal miRNA regulatory network: the upregulated miRNAs might be involved in silencing tumour suppressors and act as positive regulators of inflammatory pathways (Onco-miRs) while the miRNAs which are lower in M3 UM may have an opposite role (a tumour-suppressive role).

It has been reported that inflammation can modify miRNA expression: the level of mir-192 expression decreased in the inflammatory disease ulcerative colitis; the identified target for this miRNA was the inflammatory chemokine, macrophage inhibitory peptide (MIP)-2α (Wu 2008).

Despite the presence of immune cells in UM, the local immune responses seem to be ineffective. In order to mount an anti-tumour action, it is very important to increase the knowledge on how immune cells and especially CD8 T cells are regulated. The immune checkpoint Lymphocyte activation gene-3 (LAG3) has recently been shown to be highly expressed in UM (Durante 2020).

Immune checkpoints are cell surface inhibitory receptors that normally control autoimmune reactions in the body.

In **chapter 7**, we studied LAG3 expression in 64 primary UM samples from the Leiden cohort and verified our results using an independent cohort of another set of 15 tumours from Leiden and 80 samples from the TCGA cohort. With the knowledge that loss of chromosome 3 contributes to inflammation, we hypothesized that this cell surface inhibitory receptor would be especially expressed in M3 tumours and therefore could be used as a target for anti-immune checkpoint therapy. We found that elevated levels of LAG3 are associated with the presence of prognostically-bad markers such as an epithelioid cell type, loss of chromosome 3 and loss of BAP1 expression. Positive associations were found between LAG3 and the presence of tumour-infiltrating immune cells.

LSECtin, HLA Class II and Galectin-3 are ligands of LAG3. We found that HLA Class II molecules are highly correlated with LAG3. In addition to HLA Class I, HLA Class II expression is also linked to higher numbers of infiltrating T cells (Jager 1988) and metastasis formation (Ericsson 2001, Krishnakumar 2003). In addition to HLA Class II molecules, we also found Galectin-3 to have an increased expression in M3 tumours. This lectin has been shown to decrease the cytotoxic activity of CD8 cells (Kouo 2015).

It has been proposed that one of the mechanisms behind the exhaustion of CD8 cells is the upregulation of LAG3 ligands on the tumour, attracting LAG3-positive infiltrating lymphocytes into the tumour microenvironment and transmitting inhibitory signals to them (Durante 2020, Figueiredo 2020).

As especially high-risk UM carry high numbers of tumour-infiltrating lymphocytes (TILs) and tumour-associated macrophages (TAM), the high LAG3 distribution in these tumours suggests a potential benefit of anti-LAG3 monoclonal antibodies as adjuvant treatment in patients with high-risk UM.

Conclusions and future perspective

In this thesis, we focus on the role of different factors that may enhance the inflammatory phenotype in UM and we investigate how an anti-tumour immune attack might be disabled in this disease. We aim to increase the knowledge of the pathogenesis of inflammation in order to define possible new therapeutic targets.

Inflammation is a protective strategy of the body which normally shuts down after healing a wound. One-fourth of all cancers are caused by chronic inflammation (as reviewed by Coussens 2002); chronic inflammation fails to heal the tumour (Dvorak 1986). Inflammation is associated with the development of metastasis in UM. In recent years, different studies have identified several genetic prognostic markers highly associated with inflammation, including chromosome aberrations such as loss of chromosome 3, extra copies of chromosome 8q gain, as well as mutations, causing loss of BAP1 expression. This information classifies tumours into different risk subtypes and could help to identify patients who might be candidates for neoadjuvant trials and frequent screening. It is important to increase knowledge concerning different molecular factors related to these genetic abnormalities to understand why inflammation occurs in this disease.

Our results show that many different mediators are collectively dysregulated in inflamed tumours; upregulation of HLA-Class I was associated with upregulation of NFkB pathway components, with upregulation of a specific group of HDACs and with several specific miRNAs. All of these are upregulated in high-risk M3/BAP1-negative tumours. An exception is HDAC11, which is located on chromosome 3, and is downregulated in tumours with loss of chromosome 3.

The deregulation observed in different epigenetic modulators and the NFkB pathway might be connected: for example, the inhibition of HDAC enzymes in non-small cell lung cancer reduced NFkB transcriptional activity (Imre 2006); moreover, mir-155 which we found to be increased in M3 tumours with positive correlation with HLA Class I, is shown to be up-regulated by NFkB in lung cancer (Chiu 2016).

Based on the results of this, we hypothesise that loss of chromosome 3/BAP1 expression induces HLA expression through upregulation of the NFkB pathway and epigenetic reprogramming. The latter involves HDACs and miRNA to recruit infiltrating macrophages and lymphocytes to the

microenvironment, exhausting their cytotoxic activity via LAG3/HLA Class II or Galectin-3 interaction and at the same time using immune cells as a source of growth factors. This leads to more inflammation in the microenvironment and increases angiogenesis and subsequently metastatic spreading.

One central regulator may be the presence of ischemia. Jehs (Jehs 2014) showed that stimulation of UM cell lines led to the secretion of monocyte-attracting cytokines, while Bronkhorst proposed that it was the presence of ischemia which led to the production of pro-inflammatory cytokines (Bronkhorst 2014). Brouwer (Brower 2019) recently showed that ischemia is seen more often in UM with M3/loss of BAP1. It is interesting to see that drugs that interfere with a very important ischemia-induced cytokine, namely HIF-1 alpha, are being developed and tested for UM (Dong 2019).

Many questions remain, such as the question of whether chromosome 3/BAP1 loss and subsequent increases in HLA Class I expression on the tumour cells lead to the attraction of immune cells. Or is it the inflammatory microenvironment that upregulates HLA Class I expression and enhances genomic instability in UM? Understanding the main reason for the presence of immune cells in the eye is critical to understand the pathogenesis of this disease to be able to design proper anti-inflammatory strategies.

We hope our attempts have been useful towards the understanding of the regulation of inflammation in uveal melanoma and that it will benefit survival of UM patients.

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