# CHAPTER 1

GENERAL INTRODUCTION

# MELANOMA

Melanoma is a malignancy that arises from melanocytes, the pigment-producing cells that can be predominantly found in the eye or the epidermal basal layer of the skin. While the aetiology of this aggressive cancer can be variable, the genetic damage that drives the oncogenic transformation of the melanocytes is often induced by UV-radiation.[1;2] Pigmentation of the skin protects against this UV-induced DNA damage and it is therefore not surprising that melanoma is particularly common amongst the poorly pigmented Caucasian population.[3-5]

Mainly due to increased UV exposure, the incidence of melanoma has doubled worldwide over the past three decades (200000 new cases in 2008).[3] Primary melanomas can be easily treated by surgical resection, leading to a good prognosis for stage I patients. However, metastasized melanoma is almost completely resistant to therapeutic modalities such as radio- and chemotherapy, resulting in a median overall survival of less than one year for this patient group.[6;7]

Despite considerable efforts, for over 20 years there was no melanoma treatment developed that could improve survival of stage IV patients. However, the treatment of unresectable metastasized melanoma has progressed markedly in recent years due to the development of both immunotherapies that stimulate anti-tumor immunity and targeted therapies that block oncogenic proteins.[8;9] Two of such treatments, the anti-CTLA-4 monoclonal antibody (mAb) ipilimumab and the targeted drug vemurafenib, have shown in phase III clinical trials to improve the mean overall survival of metastasized melanoma patients.[10-12] These promising results boosted the development of many more targeted therapies and immunotherapies.

This thesis will focus on pre-clinical work concerning the optimization of melanoma treatment. In detail, it will address for both targeted therapies and immunotherapies factors that play a role in the identification of response-predictive biomarkers, the toxicity of treatments, and the potential efficacy of combination treatments.

# MELANOMA T-CELL BASED IMMUNOTHERAPY

## Rationale

Melanoma is believed to be an immunogenic malignancy since spontaneous tumor regressions have been reported and melanoma-reactive T-cells can often be found in the blood of patients.[13-15] T-cells are part of the adaptive immune system and can help to eliminate pathogens by using their T-cell receptor (TCR) to recognize non-self antigens that are presented to them by Major Histocompatibility Complex (MHC) molecules on the cell surface. Upon antigen recognition and proper co-stimulation, $CD8^+$ (cytotoxic) T-cells will become activated and can eliminate the antigen-expressing cells by releasing cytotoxins such as perforin and granzyme. T-cells specific

for melanoma-antigens can eliminate tumor cells and such an anti-tumor response is often long-term since the tumor-specific T-cells are maintained as memory cells. To evade an anti-tumor immune response melanoma cells often use different escape-methods, such as MHC downregulation or upregulation of surface molecules that inhibit the function of T-cells.[16]

Conceptually it is possible to distinguish three types of immunotherapy that can lead to a systemic anti-tumor T-cell response. First, vaccination can be used to initiate or potentiate an endogenous immune response by providing tumor antigens. Second, the adoptive transfer of (ex vivo expanded or genetically modified) tumor-reactive T-cells can facilitate an effective T-cell response towards the tumor. Third, the administration of molecules (most often antibodies) that can either directly stimulate T-cells or prevent their inhibition can improve the efficacy of tumor-specific T-cells. As clinical successes using therapeutic vaccination against shared antigens have been limited so far, we will mainly focus on adoptive T-cell transfer treatments and T-cell checkpoint alteration by immunomodulatory antibody therapy.

### T-cell receptor gene therapy

The adoptive transfer of tumor-reactive T-cells can be achieved by two different techniques. First, tumor-infiltrating lymphocytes (TILs) can be isolated from an excised tumor, expanded ex vivo to large numbers and subsequently re-infused into the patient. This TIL treatment has shown promising results in melanoma patients as objective response rates ranged between 33 and 72% in multiple clinical trials.[17-20] However, a disadvantage of this treatment is that its utilisation depends on the presence of pre-existing tumor-reactive T-cells in the tumor of the patient.

In contrast, a second technique, named TCR gene therapy, can be applied for a higher number of patients as it circumvents this requirement of pre-existing anti-tumor T-cells. The principle of TCR gene therapy is based on the fact that the TCR is the sole determinant of the antigen-specificity of a T-cell. The isolation of the genes that encode a tumor-specific TCR and subsequent transfer of these genes into the patient's T-cells can lead to the generation of a large pool of endogenous tumor-reactive T-cells.[21] It has been shown in numerous pre-clinical murine studies that these TCR gene modified T-cells can persist upon transfer, home to the tumor-site, break immunological tolerance towards a self-antigen and effectively kill tumor cells that express the antigen of choice.[22-29] Encouraged by these promising pre-clinical results, several clinical trials have been performed to demonstrate the feasibility and potential of TCR gene therapy as a melanoma treatment, showing objective response rates up to 45%.[30-32] Compared to the diverse repertoire of specificities in TIL treatment, the gene modified T-cells are only clonal (concerning the introduced TCR) and in the future probably multiple antigens will be targeted simultaneously by using multiple TCRs. Most likely, the efficacy of TCR gene therapy could be further increased when the

treatment is combined with strategies to improve functioning of the transferred T-cells such as immunomodulatory antibody therapy or manipulation of the cytokine milieu.

### T-cell checkpoint blockade antibody treatments

Another type of immunotherapy is T-cell checkpoint blockade, such as the targeting of CTLA-4 or PD-1 by monoclonal antibody treatment. Both cytotoxic T-lymphocyte associated antigen 4 (CTLA-4) and programmed death receptor 1 (PD-1) are inhibitory receptors that are located on the surface of activated T-cells. Stimulation of these receptors by their ligands (respectively CD80/CD86 and PD-L1 on the surface of APCs, but also tumor cells in the case of PD-L1) leads to a diminished T-cell function by various mechanisms such as impairing proliferation and cytokine production.[33-37] Consequently, CTLA-4 and PD-1 signaling can help to control immune responses and mediate peripheral tolerance. Tumors can inhibit tumor-reactive T-cells by stimulating these receptors, but these immune escape mechanisms can be blocked by mAb treatment directed against these co-inhibitory molecules or their ligands.[38;39]

It has been shown in a phase III study that the blockade of CTLA-4 signaling by ipilimumab, a fully human mAb against CTLA-4, leads to an improved median overall survival and objective response rate compared to gp100 vaccination (10.1 vs. 6.4 months and 28.5 vs. 11%).[11] Grade 3 or 4 immune-related adverse events (irAEs) occurred in 10-15% of patients receiving ipilimumab. The irAEs most often affected the skin and the gastrointestinal tract, but were clinically manageable by the administration of corticosteroids.

Moreover, recent phase 1 trials in patients having various solid tumors showed objective response rates in 6-17% of patients treated with anti-PD-L1 mAb and 20-25% of patients treated with anti-PD-1 mAb.[40;41] Compared to anti-CTLA-4 mAb treatment, the clinical experience with PD-1/PD-L1 mAb treatment is still rather limited and the maximum tolerable dose (MTD) has not been reached for the latter treatments yet. Perhaps related to this, anti-PD-1/PD-L1 mAb treatment seems to lead to far less severe and less frequent immune related adverse events.

While both CTLA-4 and PD-1 are inhibitors of T-cell activity, their function is not completely overlapping. Therefore, combining these T-cell checkpoint blockade treatments can result in synergy as has been shown in vivo.[42] However, this combination therapy could also increase the risk of autoimmunity. Apart from being combined with each other, these T-cell stimulating treatments could also be combined with other treatment modalities such as targeted therapy, which can sensitize tumor cells to an immune attack.

1

# MELANOMA TARGETED THERAPY

## MAPK and PI3K signaling pathway in melanoma

Mutational profiling of melanoma has helped us to gain a much better understanding of the aetiology of this malignancy. As most of the recurring mutations play a role in either the mitogen activated protein kinase (MAPK) or the phosphatidylinositol 3 kinase (PI3K) pathways it became evident that these signaling pathways have a central role in the pathophysiology of melanoma.[43-46]

The MAPK pathway consists of the kinase cascade RAS-RAF-MEK-ERK and drives tumor cell proliferation, but is also involved in differentiation and survival.[47] It is the most commonly activated signaling pathway in melanoma, mostly due to oncogenic gain-of-function mutations in BRAF ($BRAF^{V600E}$ missense mutation in 45-60% of melanomas) or NRAS (in 20-25% of melanomas).[45;48] The $BRAF^{V600E}$ mutation can lead to a 500-fold increased activity of the BRAF protein, stimulating constitutive activation of MEK/ERK signaling in tumor cells in the absence of extracellular growth stimuli.[49] This mutation is not only present in the majority of melanomas, but can also be found in other solid tumors such as papillary thyroid, colorectal, ovarian, breast and lung cancers.[50;51] These cancer types, like melanoma, are also known to often harbour RAS mutations, with the two mutations occurring in a mutually exclusive fashion.[45]

Activation of the PI3K pathway leads to signaling through the kinase AKT and subsequently mTOR. The activity of these two signaling proteins promotes cell proliferation and the survival of tumor cells by preventing apoptosis induction.[52] PTEN is a tumor-suppressor which counteracts PI3K pathway activity by dephosphorylating the signaling protein PIP3. The function of PTEN is often lost in melanoma (in around 30%) as well as in many other types of solid malignancies, leading to the activation of the PI3K signaling pathway in tumor cells.[45;53]

## Targeting the MAPK pathway

As the oncogenes that are major players in melanoma development are identified, the pool of therapeutic targets for drug development increases. Since the inhibition of signaling proteins can result in serious toxicity, the ideal oncogenic protein to block by targeted therapy is (over-) expressed exclusively in malignant cells. For melanoma treatment many different small molecule inhibitors acting on either the MAPK or PI3K pathway are currently in development, but most of them are still in the clinical testing phase and struggle with dose-limiting toxicities as they target non-mutated proteins.[54]

Considering the high incidence of the $BRAF^{V600E}$ mutation in melanoma and its tumor-specific expression, it is not surprising that the first targeted therapy to show an improved median overall survival for metastasized melanoma patients was the $BRAF^{V600E}$ inhibitor vemurafenib (also termed PLX4032).[10] Vemurafenib (and later also the BRAF inhibitor dabrafenib) has shown remarkable clinical activity with treatment

response rates of 50-60% and a median time to response of only 1.5 months.[10;55] However, these fast-developing responses are generally of a short duration (progression free survival of 5-6 months) resulting in almost all treated patients eventually relapsing due to tumor escape. Several mechanisms of BRAF inhibitor resistance have been postulated. These include bypass signaling via ARAF and CRAF or activation of the MAPK pathway via mutated NRAS or the agonist COT.[56-59] Apart from reactivation of the MAPK pathway, alternative or additional signaling via the PI3K pathway also appears to promote resistance.[60] While a single unifying resistance mechanism is lacking, most have in common that they induce (independently of BRAF signaling) activation of ERK, often through mutations that converge on the activation of the upstream kinase MEK.

## BIOMARKERS FOR TARGETED THERAPY AND IMMUNOTHERAPY

As reviewed in *chapter 2*, the development of targeted therapies and immunotherapies, such as vemurafenib and ipilimumab, will dramatically change the treatment of metastasized melanoma after many years of stasis. However, this positive development literally comes with a price as these treatments respectively cost 102.000 euro per year and 21.000 euro per course. In addition, not all treated patients will respond to the offered treatment, leading to a considerable proportion of the patients receiving superfluous treatment that can impair their quality of life due to treatment-related side-effects. To be able to predict which patients can benefit from these costly treatments, the identification of biomarkers related to treatment response will be necessary. For targeted therapy such biomarker identification will most likely be possible when extensively analysing the genetic profile of tumors prior to treatment as this may be related to the ability of a tumor to escape from the targeted therapy. However, such extensive genetic profiling is costly and labour-intensive and therefore not yet standardly applied.

For immunotherapeutic strategies there is a great need for response-predictive biomarkers as well, but despite considerable effort only very few have been postulated so far. The results of the recent phase I clinical trial studying anti-PD-1 mAb treatment suggested a relationship between PD-L1 expression on tumor cells and the occurrence of an objective response.[41] If this correlation can be validated using larger patient cohorts, unnecessary treatment of patients harbouring PD-L1 negative melanomas will be avoided. To implement the use of such a biomarker, the patient's tumor material should be evaluated for PD-L1 expression prior to the start of treatment. However, as described in *chapter 3*, PD-L1 immunohistochemistry is often not properly validated and sensitivity of the staining can be lost when using formalin-fixed paraffin-embedded instead of frozen material. Furthermore, the expression of

PD-L1 in the tumor can differ depending on location and whether the primary tumor or satellite, in-transit, lymph node or distant metastasis is analyzed. These findings should be taken into consideration when evaluating the use of PD-L1 staining as a biomarker for anti-PD-1 or anti-PD-L1 mAb treatment efficacy. In addition, *chapter 3* describes an insignificant trend towards a better prognosis for melanoma patients expressing PD-L1 in their tumor. Perhaps the expression of PD-L1 correlates to the presence of an anti-tumor immune response.

## SAFETY AND TOXICITY OF TARGETED THERAPY AND IMMUNOTHERAPY

Especially immunotherapies which stimulate T-cell responses in an unspecific manner, such as PD-1/PD-L1 blockade, can induce autoimmunity. However, autoimmunity is also observed upon immunotherapeutic interventions that are directed against specific tumor antigens and careful selection of the treatment-target is therefore important. To assure the effectiveness and safety of TCR gene therapy the target-antigen ideally meets the following criteria.[61] First, the targeted protein should preferably play a role in supporting tumorigenesis as this will limit the chance of a tumor treatment-escape by switching off the expression of the protein. Second, to minimize on-target toxicities, the expression of the targeted protein should be limited to the tumor or non-essential/immune-privileged tissues. Finally, the targeted antigen should be presented by tumors of many different patients as this will increase the number of patients for which this TCR can be used.

There are only very few TCR gene therapy targets that meet all these requirements. For example, the protein Nodal was believed to be expressed by the majority of melanomas as it seemed to be essential for melanoma tumorigenicity.[62;63] Since Nodal was also supposed to be only expressed in embryological tissues, it seemed to be the ideal target for TCR gene therapy. However, in *chapter 4* we show that although Nodal is often expressed in melanoma it is also expressed in renal tissue, raising serious questions concerning the safety of Nodal as a target for TCR gene therapy. Furthermore, we showed that there is no correlation between the expression of Nodal during the course of disease and survival. In addition, we found that Nodal expression can be lost during the course of disease and therefore we concluded that the role of Nodal in melanoma progression may be less prominent than previously suggested.

Targeting of an antigen like Nodal by TCR gene modified T-cells can result in serious on-target autoimmunity.[61] However, TCR gene therapy can also lead to the induction of off-target autoimmunity.[64;65] After introducing tumor reactive TCR genes into the T-cells, the endogenous TCR chains can pair with the introduced $\alpha$ and $\beta$ chains leading to the formation of so-called mixed TCR dimers. T-cells expressing these mixed dimers can be self-reactive and can therefore lead to autoimmunity.

In *chapter 5* we show that such off-target toxicity indeed can occur and leads to pathology similar to graft-versus-host disease. Adjustments in the design of gene therapy vectors and target T-cell populations can partly reduce the risk of the TCR gene therapy-induced autoimmune pathology by reducing the chance that TCR gene modified T-cells form a TCR which is self-reactive.[66-68]

Potential toxicity issues play a role when analyzing the efficacy of targeted therapies as well. Most targeted therapies for melanoma mediate the inhibition of an oncogenic protein in the MAPK or PI3K pathway. As these signaling proteins are also important for the functioning of non-malignant cells, such treatments often have toxic side-effects leading to necessary dose-reductions which directly affect the anti-tumor efficacy of these treatments. In *chapter 6* we show in a murine model of melanoma that MEK-inhibitor treatment leads to severe skin toxicity. Decreasing the dose reduces the incidence and kinetics of the skin toxicity, but also decreases the anti-tumor effect of the treatment. Surprisingly, combining MEK-inhibitor with BRAF-inhibitor treatment decreases the skin toxicity while sustaining the anti-tumor effect of the treatment. This suggests that MEK-inhibitor treatment could be dosed at effective levels in the absence of dose-limiting skin toxicity when combined with BRAF-inhibitor treatment.

## COMBINING TARGETED THERAPY AND IMMUNOTHERAPY

When studying the characteristics of clinical responses upon targeted therapy or immunotherapies some crucial differences become apparent. Responses upon immunotherapeutic interventions, such as anti-CTLA-4 mAb treatment, are often long-lasting, but they generally occur in only a small proportion of treated patients.[11;12;69;70] On the other hand, targeted therapies tend to lead to fast responses in the majority of the patients, but unfortunately responses are not durable in many patients.[10;54;55] Based on the diametric properties of targeted therapies and immunotherapies with respect to response rate and response duration it is thought that their combination will induce treatment synergy.

As there are many different types of targeted therapies and immunotherapies, clinical evaluation of all possible treatment combinations is not feasible. Therefore, a mouse model of human melanoma would be a substantial asset as pre-clinical testing would be possible. In *chapter 7* we describe such a murine model of melanoma. Tumors from these inducible Tyr::CreER$^{T2}$;PTEN$^{F-/-}$;BRAF$^{F-V600E/+}$ mice harbour the BRAF$^{V600E}$ mutation and the loss of PTEN expression, genetic lesions commonly found in human melanoma. Furthermore, as the mouse model is transgenic, tumors develop in the context of a tumor micro-environment while mice are fully immunocompetent, making this model valuable for simultaneously testing immunotherapies and targeted therapies.

Using this mouse model, it is possible to pre-clinically assess the effect of BRAF-inhibitor treatment on the presence of immune cells in the tumor and to determine whether the addition of anti-CTLA-4 mAb treatment improves tumor growth control. In *chapter 8* we show that treatment of $BRAF^{V600E}$/PTEN-deficient murine melanomas with a BRAF-inhibitor leads to decreased frequencies of tumor-resident immune cells, which could not be restored by the addition of anti-CTLA-4 mAb treatment. Furthermore, this treatment combination did not result in enhanced tumor control, while anti-CTLA-4 treatment did improve the effect of tumor-vaccination in B16F10-inoculated mice. These results suggest that BRAF-inhibitor treatment may negatively affect tumor-resident immune cells and therefore may hamper the induction of an effective anti-tumor immune response.

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