Preclinical optimization of melanoma treatment

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CHAPTER 9

SUMMARIZING DISCUSSION

After many years of stasis, the treatment of metastasized melanoma is now rapidly improving, as demonstrated by clinical phase III trials showing that both targeted therapy and immunotherapy are able to increase progression-free and overall survival of melanoma patients.[1-6] This thesis focuses on the pre-clinical optimization of melanoma treatment. To provide a framework for the studies described in this work, I will first discuss current directions and issues concerning the identification of response-predictive biomarkers. Subsequently, I will focus on the toxicity of both targeted therapies and immunotherapies and I will conclude by discussing the potential efficacy of combination treatments.

BIOMARKERS FOR TARGETED THERAPY AND IMMUNOTHERAPY

Current status of immunotherapy and targeted therapy biomarkers

Immunotherapeutic interventions generally lead to low response rates, while short response durations form a problem in the field of targeted therapies. Therefore, considerable efforts have been made to identify biomarkers that can help to select patients which are likely to respond to these costly treatments. Up until now such biomarker screens have been rather unsuccessful.

The identification of immunotherapy biomarkers is hampered by both the lack of harmonization in assays between different labs and the low clinical response rates, which limits the chance of finding markers which correlate to response.[7] For instance, despite extensive efforts very few biomarkers related to the effect of ipilimumab (the most advanced immunotherapy for melanoma) have been identified so far. To date, the only baseline marker that seems predictive for a treatment-response is the presence of high expression of immune-related genes in the tumor prior to treatment, but this observation could not be confirmed in a subsequent study (Haanen and Blank, unpublished data).[8] Furthermore, a low frequency of $Ki67^{+}EOMES^{+}CD8^{+}$ T-cells in the blood at baseline can predict a relapse of the patient.[9] While these are promising findings, such potential biomarkers should be validated in prospective studies before they can be considered as clinically useful.

For targeted therapy the frequent development of treatment resistance forms a great problem. Recent studies focused on determining the mechanisms by which tumors can escape treatment which resulted in the identification of many different mutations that can drive escape.[10-14] Most likely, genetic profiling of large patient cohorts will help to find a correlation between the pre-existing mutations in the tumor and the development of treatment resistance. Such a mutation screen of the tumor could also be valuable when assigning patients to combinations of targeted therapy to prevent treatment escape.

Future directions and considerations

Currently, many studies focus on the identification of prognostic and response-predictive biomarkers, but such biomarker screens can lead to contradictory results.[15;16] For example, high expression of PD-L1 on melanoma cells was reported to be associated with a low overall survival rate.[16] However, in *chapter 3* we have shown a trend towards a better overall survival for patients who express PD-L1 in their melanoma. Interestingly, PD-L1 expression has been associated with a poor prognosis for patients with other types of solid tumors. Perhaps in the case of melanoma the expression of PD-L1 indicates the presence of an anti-tumor immune response which can help to eliminate the tumor cells (despite PD-L1 expression), leading to an improved overall survival.

To improve biomarker identification and to avoid opposing results the following issues should be taken into account in biomarkers-screens. First, when studying response-predictive biomarkers in absence of an untreated control-group it is important to realize that in some cases a potential biomarker for treatment-response is actually a prognostic marker for survival. In those cases it seems that treated patients who are positive for the biomarker have an improved survival, but these patients already had a better prognosis from the start, unrelated to treatment.

Second, the study described in *chapter 3* shows that when immunohistochemistry is used in biomarker-screens, a validation of the assay by Fc-blockade and isotype staining is required. As, unfortunately, many studies fail to perform such a validation, biomarkers screens often lead to conflicting findings.[15;16]

Third, our study shows that the expression of biomarkers, such as PD-L1, can differ depending on whether you analyze the primary tumor or satellite, in-transit, lymph node or distant metastases of the same patient. This shows that a tumor is quite heterogeneous, also concerning the expression of possible biomarkers. In addition, the expression of a biomarker can change during disease progression as has been shown for the expression of Her2/neu in breast cancer.[17] Therefore, it is crucial to realise that many patients will be falsely classified as being negative for the expression of potential biomarkers when results are based on only one tumor biopsy. Analyzing multiple sites of one tumor and preferably also multiple tumors per patient will reduce the incidence of false-negative screening-results and therefore will benefit the identification of new biomarkers.

Moreover, mutational analyses will become increasingly important for biomarker-screens. Only recently efforts have been made to start to determine how often mutations coincide in melanomas.[18;19] Such information can help to categorize melanoma patients into different subsets based on the combination of their mutations. Using this sub classification of the patients, it may then be possible to identify correlations with prognosis and response to (combined) treatments. In line with this idea and realizing that a lot of work in this direction is still required, cancer treatment in the future could be based on the genetic profile of the tumor and not the tissue of origin.

SAFETY AND TOXICITY OF TARGETED THERAPY AND IMMUNOTHERAPY

Immunotherapy

For most types of immunotherapy the induction of autoimmunity is the most frequent side-effect, especially for treatments that stimulate immune-responses in a non-specific manner such as anti-CTLA-4 mAb treatment.[1;4-6;20;21] These immune related adverse events (irAEs) can range from the frequent but rather innocent occurrence of vitiligo to grade 3 rash or even grade 4 colitis. However, using corticosteroids, most of these irAEs are clinically manageable without affecting the anti-tumor efficacy of the treatment. The potential off-target toxicity of TCR gene therapy that is described in *chapter 5* could prove to be much more difficult to manage. In this study we showed that TCR gene modified T-cells can form mixed TCR dimers consisting of paired introduced and endogenous TCR chains, leading to a pool of self-reactive T-cells that can induce autoimmunity which can even be lethal for example by inducing bone-marrow failure.

To date, multiple clinical trials using TCR gene modified T-cells have been performed and none of them reported off-target toxicity similar to that seen in our murine study.[22-24] As a result, it has been suggested that this type of toxicity only occurs in mice and should not lead to problems in the human setting.[25] Arguing against this notion, it has already been shown that mixed TCR dimers can be formed in TCR gene modified primary human T-cells as well and that these cells can display auto-reactivity in vitro.[26] The optimization of TCR gene therapy protocols (e.g. by combining treatment with T-cell checkpoint blockade treatment) will lead to an improved function of TCR gene modified T-cells. If no steps are taken simultaneously to prevent the formation of auto-reactive mixed TCR dimers, it is likely that at one point TCR gene therapy off-target toxicity will be observed in the human setting as well.[27] Therefore, strategies that can limit the formation of mixed TCR dimers should be routinely applied for human TCR gene therapy. The introduction of an additional interchain disulphide bond into the TCR and the use of murine constant domains in the TCR will improve the pairing of the exogenous TCR chains.[28-30] Furthermore, the knockdown of the expression of the endogenous TCR chains by use of miRNAs prevents the formation of mixed TCR dimers (Mario Bunse, personal communication). In addition to preventing off-target toxicity, such modifications can possibly also lead to improved anti-tumor efficacy of the TCR gene modified T-cells.[27]

Another type of toxicity that can be observed with TCR gene therapy is the so-called on-target toxicity, which can occur when the targeted antigen is also expressed by non-malignant cells. A clinical trial in which carcinoembryonic antigen (CEA) was targeted by TCR gene modified T-cells led to a severe transient inflammatory colitis as the antigen was also presented by a variety of epithelial cells throughout the gastrointestinal tract.[31] In addition, antigen receptor engineered T-cells targeting

carbonic anhydrase IX induced liver toxicity since the antigen was unexpectedly also presented by bile duct epithelial cells.[32] Such toxicities can be avoided when carefully assessing the expression pattern of any new target for TCR gene therapy.

In *chapter 4* we describe such an expression-screen for the protein Nodal as we were planning to target this protein by TCR gene therapy. For this screen we initially used a tissue-array containing 33 different types of tissue. This immunohistochemical assay demonstrated the expression of Nodal in the kidney, which was confirmed in additional renal tissue. Therefore we concluded that targeting of this protein could result in renal toxicity. In future expression-screens we would propose to combine immunohistochemical screens with gene expression data as it will be possible to screen a larger amount of tissues in this way. In addition, screening for in vitro reactivity of TCR gene modified T-cells against a panel of human tissue cultures could be valuable. However, such assays will most likely still not be sensitive enough to detect very low expression levels of the protein of interest or expression in a very select population of cells, such as bile duct epithelial cells. Until better expression screening assays will become available, such as detailed gene-expression overviews for every cell-type, we will have to accept that the safety of a TCR gene therapy target can only be confirmed when used in the clinic.

Targeted therapy

While immunotherapeutic interventions often lead to autoimmunity, the toxicities observed in response to targeted therapy are of a very distinct nature as they are caused by inhibition of crucial basic signaling pathways. Naturally, the type of toxicity depends on which signaling protein is inhibited by the treatment. However, as most signaling pathways are heavily interconnected it can be difficult to predict toxicity as inhibiting one signaling pathway can also affect another. It seems that tyrosine kinase inhibitors that target EGFR or MEK lead to dermatologic side-effects such as acneiform rash.[33;34] Apart from skin toxicities, many targeted therapies induce fatigue and also affect the gastrointestinal tract, often leading to the occurrence of diarrhea.[3;35] The exact mechanisms responsible for these side-effects remain unclear which complicates their treatment. As a result of the lack of drugs that can counteract the toxicities, the dose of small molecule inhibitors frequently needs to be decreased which sometimes directly diminishes the anti-tumor effect of the treatment, as for example seen for MEK-inhibitors.[35;36]

In *chapter 6* we show that, similar to the human setting, treatment of mice with the MEK-inhibitor GSK1120212 leads to skin toxicity. Surprisingly, we discovered that concurrent BRAF-inhibitor treatment can delay the incidence and kinetics of this toxicity, while tumor control remains unaltered. The MEK-inhibitor induced skin toxicity has been suggested to be mediated by inhibition of ERK activity in keratinocytes.[37] However, in our study we show that concurrent BRAF-inhibitor treatment does not

restore ERK activity while it does decrease the skin toxicity. Therefore, we conclude that restoring ERK activity to baseline levels is not required to reduce this side-effect. Up until now it remains unclear how BRAF-inhibitor treatment can decrease MEK-inhibitor induced skin toxicity. Unravelling this mechanism will facilitate the development of drugs that can help to prevent this side-effect. Therefore, the effect of the small molecule inhibitors on the activation of signaling proteins in the MAPK and PI3K pathway should be monitored. As a complicating factor, we should take into account that the balance between the different signaling pathways differs per cell type. Due to the complex nature of such studies, dose reductions to prevent toxicities currently seem to be the only option even though this usually has direct implications for the anti-tumor effect of the targeted therapies.

IMMUNOTHERAPY AND TARGETED THERAPY COMBINATION TREATMENTS

Immunotherapy can lead to long-lasting responses in a small proportion of patients. Unfortunately, for some treatments, such as ipilimumab, these clinical responses need quite some time to develop and during this period many patients deteriorate.[4;20;38] In contrast, the majority of melanoma patients develop a fast, but short-lasting, clinical response upon targeted therapy such as vemurafenib.[2] Based on these diametric properties of targeted therapy and immunotherapy it is thought that their combination will induce treatment synergy. Currently the safety and efficacy of many different types of targeted therapy and immunotherapy are being tested in clinical trials. As such a clinical evaluation of all possible treatment combinations is not feasible, murine models will be required to be able to select the most promising treatment combinations that can subsequently be tested in clinical trials.

Murine melanoma model to assess the potential of combination treatments

Pre-clinical studies that focus on melanoma immunotherapies are classically performed in syngeneic transplantation models, such as the B16 model. These models have a number of important limitations that have direct implications for their use in pre-clinical testing of treatment combinations.[39;40] First, as tumors are transplanted, the tumor microenvironment is very dissimilar from the human situation, because the tumor did not co-evolve with the tumor stroma. Second, a proportion of the tumor cells usually die during the transplantation process. While enough cells will survive to establish a tumor, the debris of the dead tumor cells will act as a tumor vaccine or induce activation-induced death of the antigen-specific T-cells which could alter the effect of the immunotherapy. Finally, the syngeneic tumor cells often lack the mutations that can be found in human melanoma. This complicates the use of targeted therapies in these models. The use of human melanoma cells in a

xenotranplant setting would overcome this last limitation. However, these murine models are also not suitable for the purpose of testing treatment combinations, as these tumors can only be transplanted into immunodeficient mice and therefore immunotherapy cannot be applied.

The use of genetically modified mouse models of melanoma would overcome these limitations, but they often display a low tumor incidence and a long tumor latency as for example seen in the $Cdk4^{R24C/R24C}$ murine melanoma model.[41;42] To address these challenges we crossed and characterized, as described in *chapter 7*, $Tyr::CreER^{T2}$;$PTEN^{F-/-}$;$BRAF^{F-V600E/+}$ mice, a transgenic inducible murine melanoma model in which all animals develop a rapidly growing melanoma within one month of induction. As the tumors of these immunocompetent melanoma model mice harbour the common $BRAF^{V600E}$ mutation and the loss of PTEN, the model can be used to simultaneously test targeted therapy and immunotherapy. However, extensive use of this melanoma model revealed practical issues, such as the high frequency of spontaneously developing melanomas, but also more fundamental limitations.

First, the murine melanomas are only representative of a subset of human melanomas. As discussed previously, genetic profiling of melanomas is not routinely performed and therefore it has been unclear for a long time how often the $BRAF^{V600E}$ mutation is detected in combination with the loss of PTEN. However, in a recent large study it has been shown that around 40% of $BRAF^{V600E}$ mutated melanomas also lost the expression of functional PTEN.[18] As the $BRAF^{V600E}$ mutation occurred in 63% of the studied melanomas, it is expected that this combination of genetic lesions occurs in around 25% of melanomas. In contrast, another study by Krauthammer et al. estimated this mutational combination to occur in only 4% of melanomas.[19] Although such differences may result from disparity of the used tumor material in both studies (metastases versus primary tumors), they do demonstrate that more work is required to determine the exact incidence of certain mutations.[19] Nevertheless, most melanomas have a deregulated MAPK and PI3K pathway and in that respect the melanoma model mice do represent the majority of melanoma patients. Moreover, loss of functional PTEN has been suggested to be involved in resistance to BRAF-inhibitor treatment and this hypothesis could be further studied in our melanoma mice.

The results obtained in the murine melanoma model should not be generalized to all melanoma patients as they may not reflect human patients whose tumor has a different genetic profile. To overcome such a limitation, a panel of inducible murine melanoma models harbouring different genetic mutation profiles should be developed for testing of the efficacy of combination treatments. Such models could also be used to gain more insight into treatment resistance mechanisms, although the total number of mutations will be much lower than in the human melanomas. Based on the recent genetic profiling studies we would propose to generate murine melanoma models in which the loss of the CDKN2a or p53 gene is combined with

the $BRAF^{V600E}$ or NRAS mutation as these lesion occur most often.[18;19] However, as it remains unclear which (mutated) proteins drive the melanoma biology and treatment-response, one could wonder if murine melanomas harbouring a limited mutational profile will ever be able to closely represent the human melanoma.

A second limitation of the melanoma model may be formed by the potential limited immunogenicity of the tumors. While we observe the presence of various immune cells in the tumors, we never detected a functional anti-tumor immune response when treating the mice with different types of immunotherapy. This led us to question the immunogenicity of the tumors of the inducible murine melanoma model mice. This potential limited immunogenicity could result from that fact that the melanomagenesis in these mice is driven by two genetic lesions that lead to the formation of a tumor in the very short time span of one month. Most likely, as a result of this fast and strong process, the tumors will have a low frequency of bystander-mutations. Such mutations are important for the immunogenicity of a tumor as they lead to the presentation of many non-self neo-antigens by the tumor cells. Furthermore, pigmented cells are known to be immunogenic as they express melanoma antigens such as tyrosinase related protein 1 (Tyrp1). However, the majority of the tumor mass in these mice is usually amelanotic which could also account in part for the lack of immune responses against the murine melanomas.

A number of different strategies could be applied to improve the potential limited immunogenicity of the inducible melanomas by increasing the number of immunogenic antigens presented by the tumor. For example, similar to the human setting, UV irradiation of the skin prior to tumor induction will most likely increase the mutational load of the skin and thus the tumor, leading to a higher number of potentially immunogenic bystander-mutations.[43;44] As a second strategy, genetic modification of the melanomas using a lentivirus could induce the specific expression of chosen antigens. This strategy would be more controlled, but therefore also more artificial than the UV irradiation strategy. Finally, Damsky et al. recently showed that β-catenin stabilization as a third genetic lesion in the inducible melanomas leads to a higher degree of pigmentation of the tumors which may increase their immunogenicity as melanocyte-antigens are usually quite immunogenic.[45] Currently, we are assessing the ability of all these three strategies to enhance the frequency of immunogenic antigens presented by the tumors cells.

Testing the potential of combined vemurafenib and ipilimumab treatment

The anti-CTLA-4 mAb ipilimumab and the targeted drugs vemurafenib were recently EMA-approved. As treatments with these drugs lead to diametric response patterns, the combination of ipilimumab and vemurafenib is currently tested in a clinical trial. We decided to analyse (upfront) the combined effect of these two treatments in the inducible melanoma model mice. As described in *chapter 8*, we observed that BRAF-

inhibitor treatment led to decreased frequencies of tumor-resident immune cells, which could not be restored by the addition of anti-CTLA-4 mAb treatment. In line with this finding, the combination of these treatments did not result in enhanced tumor control, while anti-CTLA-4 treatment did improve the effect of tumor-vaccination in B16F10-inoculated mice. Although it remains unclear how BRAF-inhibitor treatment leads to reduced frequencies of tumor-resident immune cells, these data do indicate that BRAF-inhibitors may not be suitable to combine with immunotherapies. Nevertheless, these findings do not necessarily argue against a synergistic effect of ipilimumab and vemurafenib in the treatment of human melanoma as this potential synergism depends on many different factors such as the induction of immunogenic tumor cell death.

It has been shown by Wilmott and Long et al. that immune cell frequencies can increase in human melanomas when cell death is induced by the vemurafenib treatment.[46] In line with this data, Arlene Sharpe recently presented work not only showing increased numbers of $CD8^+$ T-cell in melanoma-biopsies of vemurafenib treated patients, but also demonstrating that their activity, as measured by perforin-secretion, was increased. Although the reports concerning induction of necrosis upon use of vemurafenib are sometimes contradictory, the majority of melanoma patients at least have a radiological partial (and often temporarily) tumor regression in response to treatment.[2;47;48] Therefore, in the majority of patients there will most likely be a short wave of tumor-antigen release upon start of the targeted therapy. In contrast, BRAF-inhibitor treatment of the murine melanomas led to a strong reduction in tumor outgrowth, but did not result in the induction of tumor cell death. While the exact mechanism of action of ipilimumab has not been defined yet, it has been shown in many pre-clinical studies that anti-CTLA-4 mAb treatment can synergize with treatments that lead to antigen release.[49;50] Therefore, a synergy between ipilimumab and vemurafenib treatment may be observed in patients in which the vemurafenib treatment induces tumor cell death.

BRAF-inhibitor treatment did not lead to tumor cell death and a resulting wave of antigen release in the murine melanomas (most likely due to the loss of PTEN in the tumors). In that respect, the murine melanomas seem to represent the subset of human melanomas that do not show tumor regression in response to vemurafenib treatment. Most likely the strong reduction of tumor cell proliferation in response to treatment results in decreased stromal changes in the tumor and thus decreased expression of inflammatory molecules. Consequently, this could lead to the observed decreased immune-cell frequencies in the tumor upon BRAF-inhibitor treatment. To further study the exact mechanism by which tumor-resident immune cell frequencies decrease we are currently comparing the gene-expression profile of BRAF inhibitor treated murine melanomas to that of mock treated tumors.

In our study we showed that vemurafenib leads to decreased frequencies of tumor-resident immune cells. However, when there is an anti-tumor immune response

ongoing, vemurafenib could potentially stimulate this response. The group of Antoni Ribas recently showed that vemurafenib can increase MAPK signaling in activated adoptively transferred T-cells resulting in an increased in vivo cytotoxic activity and intratumoral cytokine secretion. In addition, vemurafenib treatment may support the immune system in a more indirect manner as well. A study by the group of Patrick Hwu showed that $BRAF^{V600E}$ signaling in melanoma cells leads to the secretion of IL-1 into the tumor stroma.[51] Subsequently, the IL-1 acts on the cancer-associated fibroblasts that upregulate their expression of PD-L1 and Cox-2, leading to a negative influence on the immune system. Vemurafenib treatment can reverse this process by blocking the $BRAF^{V600E}$ signaling.

Taking all these findings into account, the combination of ipilimumab and vemurafenib may show treatment synergy. Most likely this depends on whether vemurafenib treatment leads to tumor cell death and/or whether anti-tumor immune responses are present prior to treatment. Both these potential requirements for treatment synergy were not observed in our melanoma model mice which may explain our findings. A potential mechanism for treatment synergy in human melanomas could be as follows. Results obtained with TIL therapy have shown that pre-existing (tolerized) tumor-reactive T-cells potentially can contribute to an anti-tumor immune response.[52-55] Vemurafenib may stimulate their activation by inducing antigen-release. Subsequently, these activated T-cells can be stimulated by the blockade of CTLA-4 signaling and by the mentioned direct and indirect actions of vemurafenib.

Further development of combination treatments

As discussed previously, it is important to determine (pre-clinically) which treatment combinations could potentially synergize. Apart from combining different immunotherapies with each other, these treatments can be combined with strategies that induce immunogenic cell death.[56;57] Targeted therapy can induce cell death, but since the MAPK and PI3K pathway are important for the function of T-cells, these drugs often have a negative effect on these immune cells.[58;59] Therefore, most targeted therapies cannot be administered simultaneously with immunotherapies. Sequential treatment schemes could prevent a negative effect of the targeted therapy on the T-cells and will lead to waves of antigen release, as in vaccination prime-boost settings. While exploring such treatment protocols, we for example observed that the frequency of tumor-resident immune cells restored rapidly (after 7-14 days) upon halting BRAF-inhibitor treatment (unpublished data). Future research should focus on the interaction between targeted therapies and the immune system in order to determine if a sequential treatment protocol could be useful. In addition, the combination of immunotherapeutic interventions with radiotherapy is a promising line of research as radiotherapy will not systemically affect the immune system and has already shown to lead to positive results in vivo.[50;60]

SUMMARY

Biomarkers

The development of new immunotherapies and targeted therapies for the treatment of human melanoma represents a big step forward in improving the prognosis of metastasized patients. However, a major disadvantage of melanoma treatment at the moment is that many patients receive a costly and potentially harmful treatment which will not be beneficial to them. To further optimize melanoma treatment it will be important to identify biomarkers that are predictive for a treatment response. Preferably, such biomarker screens should be performed on large patient populations, should use validated screen-assays and should take into account the heterogeneity of most tumors. In addition, we expect that the genetic profiling of tumors will be a very valuable tool in the identification of predictive biomarkers. Upon identification, potential biomarkers should be validated in prospective studies before being used in a clinical setting.

Toxicities

Most cancer treatments lead to some form of toxicity. Especially the immunotherapies that stimulate the immune system in a non-specific manner can induce autoimmunity. However, using corticosteroids this type of toxicity is usually manageable. Of all discussed immunotherapies, it seems that the toxicity of TCR gene therapy is the most unpredictable and potentially most harmful and therefore strategies that improve the safety of this treatment, e.g. by preventing the formation of mixed TCR dimers, are required. Currently, the mechanisms associated with targeted therapy toxicities and drug-efficacy are still mainly unknown. To advance these treatments unravelling these mechanisms will be a necessary step as most targeted therapies cannot be dosed effectively at the moment due to dose limiting toxicities and since most treatments do not induce long-term disease control.

Optimizing treatment combinations

Murine models of melanoma form a crucial tool in the pre-clinical optimization of melanoma treatment, especially when studying the potential of combination treatments. To increase the translation of findings in murine melanomas to the human setting it is important that melanoma models resemble melanoma patients as much as possible. First, melanoma is known to be an immunogenic malignancy and therefore the murine tumors need to be immunogenic to some extent as well. Second, the genetic profile of melanomas can be quite diverse and therefore additional murine models that harbour different combinations of mutations in their tumors should be generated. Screening of treatment effects should be performed in these different melanoma models as this may increase the translation of results to the human setting. However, human melanomas of course harbour many more mutations which may be important for the biology of the tumor as well.

As for most cancers, combination therapies will form the future of melanoma treatment. Most likely cell death inducing agents will be combined with multiple treatments that can stimulate anti-tumor immune responses. Pre-clinical studies, such as those described in this thesis, will be crucial for this optimization of melanoma treatment.

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