Improving immunotherapy for melanoma: models, biomarkers and regulatory T cells

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Chapter 7

Outlook

Despite recent advances in the clinic, a subset of melanoma patients fail to respond to currently available therapies. In chapter 1 of this thesis, I have focused on the patient subgroup that remains unresponsive to treatment with anti-PD-1 and anti-CTLA-4. Nonetheless, it is imperative to note that an alternative form of immunotherapy using adoptive transfer of T cells (ACT), has been recently established for patients with late-stage melanoma. In a recent phase III trial testing ACT in patients with unresectable stage IIIc and stage IV melanomas, 7.2 months- and 25.8 months-long median progression-free and overall survival, respectively, were achieved [1]. Even upon treatment with ACT, however, 51% of the patients lack an objective response [1]. Furthermore, ICB therapies have been approved as first line of treatment for nearly 14 solid tumor types [2]. PD-1 blockade therapies are associated with high response rates in cancer types such as desmoplastic melanoma, Merkel cell carcinoma and MSI-high colorectal cancer, with an objective response rate (ORR) of 70%, 56% and 53%, respectively [3]. On the other hand, the ORR is as low as 15% in tumor types such as head and neck cancer, gastroesophageal cancer and bladder and urinary tract cancer [3]. Thus, novel therapies (either alone or in combination with existing immunotherapies) are essential for the non-responding subgroup of cancer patients.

Among the strongest determinants of melanoma prognosis and response to immunotherapies are the infiltration by, and activity of, $CD8^+$ T cells [4,5]. However, several factors in the tumor including loss of tumor antigen, defects in antigen processing and presentation machinery, and insufficient priming by dendritic cells prevent the successful activation and recruitment of $CD8^+$ T cells into the tumor [6]. Furthermore, chronic activation in the TME causes exhaustion/dysfunction of $CD8^+$ T cells and hamper the efficient killing of tumor cells [6]. Additionally, the presence of suppressive regulatory T cells (Tregs) and metabolic restrictions can dampen anti-tumor CD8 responses [6]. Therefore, enhancing responses to immunotherapy requires a shift in the balance of anti-tumor immunity towards increased $CD8^+$ T cell functionality and reduced suppression in the tumor microenvironment (TME). In this thesis, I have first addressed the need for syngeneic murine melanoma cell lines that can be used for pre-clinical research aiming to enhance responses to immunotherapy in melanoma (chapter 2). Thereafter, I have evaluated two approaches to enhance anti-tumor immune responses:

i. Use of class specific HDAC inhibition, which could be combined with immune checkpoint blockade (ICB) to increase the efficacy of therapy (chapters 3-4).
ii. Obtain an increased understanding of the metabolic advantage of Tregs in conditions of the TME with an aim to decrease Treg-mediated suppression in the tumor (chapters 5-6).

As described in chapter 2, our novel murine cell lines, arising from a single induced primary tumor from *Braf*^{V600E}*/Pten*$^{-/-}$ mice, recapitulate immune resistance mechanisms that commonly occur in melanoma patients. We first established the MeVa2.1 and

MeVa2.2 cell lines that had low immunogenicity thus failing to elicit an effective anti-tumor immune response. Upon stable expression of the foreign antigen ovalbumin (OVA), we observed differences in immune-mediated growth control between the two cell lines. These subtle differences between cell lines that were established from a single induced primary tumor, also sets our model apart from other cell line series which do not share the same primary tumor origin [7,8]. While we expressed ovalbumin (OVA) to render the cell lines immunogenic, the parental cell lines could be used as a platform to experiment on strategies aiming to increase immune infiltration into the tumor. Moreover, MeVa2.1.dOVA tumors displayed a heterogenous response to various forms of immunotherapy, exemplifying the non-responder sub-group of melanoma patients. Therefore, this model provides a therapeutic window to test novel combination approaches to immunotherapy. In the later part of this thesis (chapters 3, 4, 6), we have utilized the MeVa2.1.dOVA model to unravel the efficacy of combination therapies *in vivo*.

In this final chapter, I will discuss the rational use of combinations to ICB in melanoma. With a focus on the use of specific HDAC inhibition, targeting Tregs and metabolism, I will discuss recent/ongoing clinical trials in melanoma utilizing these approaches. Lastly, I have postulated the need for patient stratification using predictive biomarkers in trials evaluating combination approaches to ICB across cancer types.

Is there still light at the end of the tunnel for HDAC inhibition in melanoma?

Patients with a low IFN-γ response signature in their baseline tumor biopsies are less likely to respond to neoadjuvant ICB [9], thereby needing additional treatment combinations. Inhibiting HDACs was previously shown to improve anti-tumor immune responses [10] and to increase IFN-γ response signature in the tumor [11], thus becoming likely candidates to combine with ICB. Nonetheless, clinical trials testing the efficacy of pan-HDAC inhibitors such as vorinostat have been met with limited success due to increased toxicities [12]. The toxicities observed in early clinical trials using pan-HDACi are largely due to the broad tissue expression of HDACs and inhibition of effector T cell proliferation and cytokine production [13,14]. Both these caveats of pan-HDAC inhibition can be easily overcome by class specific HDAC inhibitors. As a result, despite repeated reports of toxicities using pan-HDAC inhibitors [12–14], the interest in HDAC inhibition has not waned. We determined whether specific inhibition of HDAC class I, using domatinostat, could enhance response to ICB in melanoma (chapter 3). The results from the MeVa2.1.dOVA murine model showed an enhanced tumor growth control and prolonged survival of tumor-bearing mice upon addition of domatinostat to anti-PD-1+anti-CTLA-4. This was due to an increased tumor-reactive $CD8^+$ T cells

and elevated IFN-γ response, thereby encouraging the use of this combination in the clinic. However, domatinostat failed to modulate immune responses or to increase the efficacy of ICB in patients in the DONIMI clinical trial (chapter 3).

One of the reasons for the failure of the DONIMI trial is attributed to the unexpected domatinostat-related skin toxicity, which prevented dose escalation in patients. Skin toxicities such as rash and pruritus are common adverse events upon treatment with ICB [15,16]. Importantly, it was the rapid progression and reoccurrence upon re-treatment, rather than the type of skin rash, that was specific to domatinostat. Lack of toxicity upon domatinostat administration in the murine model highlights one of the limitations related to translating *in vivo* findings into patients. In general, immune related adverse events (irAEs), often observed with combination ICB therapy in patients, are not present in murine models. The inherent species-related differences between mice and human, use of specific pathogen free (SPF) animals, differences in microbiome are a few of the multitude of factors that interferes with direct translation of *in vivo* safety profile of drugs into the clinic.

A starting point to address this is to decipher the type/cause of irAE in ICB-treated patients and modelling this back *in vivo* (in mice). Most of the murine melanoma models currently available are syngeneic to C57BL/6 mice and this strain suffers the least from ICB-induced irAE [17]. A recent study showed that crossing the C57BL/6 strain with the (autoimmunity-prone) MRL/lpr mice allowed for the evaluation of ICB-related inflammation in the secondary organs (lung, colon, liver and pancreas) using existing tumor cell lines [17]. Specific irAEs associated with certain murine models is exacerbated by ICB administration. Examples for such models include dextran sulfate sodium-induced colitis [18], deletion of STAT3 in dendritic cells leading to intestinal toxicity [19], repetitive injections of anti-CTLA-4 (and complete Freund's adjuvant) to induce hypophysitis in the pituitary gland [20], and colonization of the commensal bacteria *Staphylococcus epidermis*, leading to skin inflammation upon anti-CTLA-4 administration [21]. Another study highlighted a straight-forward approach by the use of older mice (18-24 months) to recapitulate the multi-organ inflammation caused by treatment with anti-PD-1 [22]. Assessing the effect of novel drug-ICB combinations in such models could provide insights into treatment tolerability, in the context of known ICB-related irAEs.

Intriguingly, the studies describing ICB treatment-induced irAE do not report obvious behavioral changes or manifestation of the inflammation into detectable symptoms or fatality in mice [17,20,22]. It is therefore likely that some irAEs might be overlooked with the conventional design of *in vivo* experiments, in which tumor growth upon drug treatment is monitored. Reflecting upon our own *in vivo* study (in chapter 3), it is difficult to conclude that the use of one or more of these approaches would have completely prevented the risk of testing domatinostat in the neoadjuvant trial. Nevertheless, including additional readouts such as monitoring the serum for signs of systemic

inflammation and evaluating immune infiltration in secondary organs at the study endpoint, in these experiments might provide key information regarding the effect of drug combinations on irAEs. While such intermediate steps between pre-clinical to clinical translation will not eliminate the use of potentially toxic drug combinations in trials, they are still useful in lowering the risks associated with increased irAEs.

Furthermore, we probed the effect of targeting HDAC class IIa, using the specific inhibitor LMK235, on Tregs and determined whether this could in turn improve tumor growth control (chapter 4). LMK235 did not show any efficacy *in vivo* alone or in combination with anti-PD-1, providing sufficient evidence to stop pursuing this compound (chapter 4). A recent study investigated the efficacy of combining class I/IV specific HDAC inhibitor Mocetinostat with ICB in treatment-naïve stage III/IV melanoma patients [23]. Mocetinostat in combination with anti-PD-1+anti-CTLA-4 increased markers associated with pro-inflammatory response, but this was accompanied by high grade toxicities in all patients [23]. In another recent trial (PEMDAC) in patients with uveal melanoma, combination of entinostat (class I HDACi) and anti-PD-1 resulted in therapeutic benefit in a subset of patients (overall response rate 14%) [24]. In this trial (with n=24 patients), entinostat dose reduction/interruption was essential due to toxicity in 31% of the patients and treatment was discontinued due to toxicity in 10% of the patients, but overall, the adverse events were reportedly manageable [24].

Nevertheless, dose-limiting toxicity and low efficacy observed using class specific HDACi + ICB in cutaneous melanoma is a cause for concern. If future trials will be conducted to test other class-specific HDACi in melanoma, these must be preceded by additional pre-clinical studies to ensure drug-tolerability in patients. For example, conducting *in vivo* studies in older mice or using mice strains prone to autoimmunity and systemic monitoring for signs of overt inflammation in existing models might provide early indications of potential immune-related toxicities. Results from an ongoing clinical trial combining class I specific HDACi, entinostat, with anti-PD-1 in patients with advanced stage melanoma (NCT03765229) are critical to ascertain whether there could be increased therapeutic benefit, alongside manageable toxicities, using this combination.

Specific targeting of Tregs in the tumor

Although Tregs are an attractive target to enhance anti-tumor immune responses, limited strategies exist in the clinic that can specifically target Tregs in the tumor. Currently available strategies target markers that are also expressed by other immune cell subsets, such as effector $CD8^+$ T cells and $CD4^+$ T cells. Moreover, systemic targeting of Tregs might result in auto-immune side effects. To address the challenges associated with therapeutic targeting of Tregs specifically in the tumor, it is essential to obtain an increased understanding of the biology of Tregs, which are described in chapters

5 and 6. Given the differences in metabolic pathways engaged by Tregs in the tumor compared with effector T cells, targeting metabolism of Tregs might be beneficial to reduce Treg-mediated suppression in the tumor (as elaborated in chapter 5).

Our findings in chapter 6 show that tumor-associated acidity augments immune-suppressive Tregs. These observations expand on existing knowledge that low pH in the tumor reduces $CD8^+$ T cell function. Targeting tumor-associated acidity increases activation and cytokine production of $CD8^+$ T cells and controls tumor growth in combination with ICB [25]. In addition, we show that increasing pH in the TME lowers Treg frequencies in the tumor (chapter 6). This highlights a potential 'win-win' situation upon neutralizing tumor pH. It is conceivable that this approach could be extended to other molecular targets that hinder anti-tumor immune responses, but are not exclusive to Tregs. Deducing another example from chapter 6, targeting the lactic acid-mediated increase in the expression of CD39 on Tregs might be beneficial. CD39 expression is not exclusive to Tregs and is upregulated also by exhausted $CD8^+$ T cells in the tumor [26]. Ongoing trials (NCT05381935; NCT04261075) will provide answers to whether CD39 blockade in patients with solid tumors can corroborate the positive outcome on anti-tumor immune responses that were earlier observed using *in vivo* murine models [27].

In recent years, several drugs targeting Tregs have entered clinical trials. Of note, Tregs are known to express CTLA-4 on their surface at higher levels compared with $CD8^+$ T cells and certain studies have shown that anti-CTLA-4 can deplete Tregs, consequently reducing suppression in the tumor [28]. However, the validity of these findings in anti-CTLA-4-treated patients has been disputed [29]. Nonetheless, modifying the Fc tail of anti-CTLA-4 can deplete Tregs through induction of antibody-dependent cellular cytotoxicity, therefore controlling tumor growth [30]. Moreover, depletion of Tregs specifically in the tumors could be achieved using intra-tumoral administration of viral vector encoding Fc modified anti-CTLA-4 and GMCSF [30], and this approach is currently being tested in combination with anti-PD-1 therapy in the clinic (NCT04725331). Another strategy to deplete Tregs is by targeting CD25 since Tregs are known to over-express this marker in the tumor [31], and this is currently being evaluated in patients (NCT04158583). However, both these markers are not specific to Tregs and are expressed also by effector T cells upon activation. Therefore, a depleting antibody runs the risk of potentially depleting activated effector T cells. The results of these trials will shed light on the selectivity of these antibodies in patient tumors and if this can increase anti-tumor immune responses. Furthermore, a recent study has described the effect of antisense oligonucleotides on directly reducing FoxP3 expression [32]. Preliminary results show reduced expression of FoxP3 *in vivo* and a consequent reduction in Treg-mediated suppression [32]. This also resulted in improved tumor growth control [32], displaying promising early evidence for this approach. Directing such therapies into the tumor might be essential to prevent autoimmune-mediated toxicities that will otherwise arise due to systemic depletion of Tregs.

I have focused on the FoxP3 expressing Tregs in this thesis, since it is known that the metabolic pathways in Tregs are governed by FoxP3 expression. It is imperative to note that 'unconventional' Treg subsets such as type I regulatory (Tr1) cells have been described [33]. Tr1 cells are a subset of $CD4^+$ T cells, that do not express FoxP3 but are capable of suppressing effector responses via secretion of soluble factors such as IL-10 and TGFβ [33]. Owing to their suppressive functions, it is important to decipher the role of Tr1 cells in the context of anti-tumor immunity, especially upon depletion of conventional $FoxP3^+$ Tregs.

Targeting components of the glycolytic pathway to enhance response to immunotherapy in melanoma

In chapter 6 of this thesis, we show that a low pH due to the presence of extracellular lactic acid supports a suppressive environment in the tumor and propose that targeting this might be beneficial in melanoma. Increased lactic acid production in the TME is attributed to the elevated glycolytic activity of tumor cells. Low glucose and high lactic acid levels support the survival, functionality [34] and induction (chapter 6) of Tregs, while forming a metabolic barrier for the proliferation and cytokine production of effector immune cells [35,36]. Melanoma patients with a high glycolytic score prior to anti-PD-1 therapy are likely to have a short progression-free survival compared to those with a low glycolytic score [36]. Moreover, the expression of genes associated with the glycolytic pathway is negatively associated not only with T cell infiltration in the tumor, but also with response to ACT in patients with melanoma [37]. Overall, the idea of therapeutically lowering lactic acid levels in the tumor could also be achieved by targeting upstream mediators of the glycolytic pathway. This is especially relevant in the context of melanoma since aberrant activation of MAPK pathway by expression of $BRAF^{V600E}$ mutation is known to increase glycolysis in tumor cells [38]. It has been shown in a neoadjuvant murine model that tumors with reduced glycolysis (using LDHA knockdown), are amenable to anti-CTLA-4 therapy and have a significantly prolonged metastasis-free survival compared with highly glycolytic tumors [39]. Therefore, targeting tumor glycolysis might augment response to ICB in melanoma, even in the neoadjuvant setting. Pre-clinical studies utilizing tumor-specific gene knockdown/knockout provides a strong rationale to combine glycolytic inhibition along with ICB in melanoma [40]. In addition, certain inhibitors used in pre-clinical melanoma models have shown promising results upon targeting various components of the glycolytic pathway in combination with ICB, as highlighted in the following paragraphs.

Metformin, used to treat type 2 diabetes, is known to reduce gluconeogenesis via increased activation of adenosine monophosphate-activated protein kinase (AMPK), thereby regulating glycolysis [41]. Treatment with metformin can reduce glycolysis in

tumor cells, predominantly by lowering glucose transporter 1 (GLUT1) levels [42]. In addition, metformin is known to impact mitochondrial respiration causing reduced hypoxia in the TME [43]. In a retrospective analysis in patients with advanced stage melanoma (n=55), treated with anti-PD-1 and/or anti-CTLA-4, metformin usage caused a trend towards longer survival, albeit statistically insignificant [41]. While it is impossible to attribute the efficacy of metformin solely to reduced glycolysis, due to its pleiotropic effects, it is still a likely candidate to combine with ICB. Along these lines, pre-clinical evidence already points out to slower B16.OVA tumor growth in mice treated with combinations of metformin and anti-PD-1 compared with either treatment alone [43].

Diclofenac is shown to inhibit the activity of monocarboxylate transporters (MCTs) 1 and 4, involved in the bi-directional transport of lactate - the end product of glycolysis [36]. Diclofenac treatment, in combination with anti-PD-1 or with anti-PD-1+anti-CTLA-4, slowed the growth of B16 melanoma tumors, which were otherwise unresponsive to either treatment alone [36]. A small molecule inhibitor of MCT1, AZD3965, has been tested in a phase I trial in patients with diffuse large B-cell lymphoma and Burkitt's lymphoma and is deemed safe [44]. Another study has ameliorated this drug, by loading AZD3965 in ultra-pH-sensitive nanoparticles, which are then released only in the acidic TME, allowing a tumor-directed targeting of MCT1 using lower drug doses [45]. Furthermore, this synergized with PD-1 blockade and lowered tumor volume and prolonged survival in B16-F10 melanoma model [45]. Future trials aiming to combine MCT inhibition with ICB in melanoma could consider the use of AZD3965.

Furthermore, small molecule inhibitors of enzymes involved in the glycolytic pathway such as the rate-limiting pyruvate kinase (PKM2) [46] and pyruvate dehydrogenase kinase (PDK) [47] have been described. Such inhibitors can reduce glycolysis and lower proliferation in melanoma cells [46,47]. However, their efficacy in combination with ICB *in vivo* in melanoma models is not yet deciphered.

Targeting glycolysis systemically could act as a double-edged sword by impacting $CD8^+$ T cells, that are known to engage glycolysis for effector functions. Contrary to this idea, treatment with diclofenac, which inhibits lactic acid production by tumor cells, resulted in increased cytokine production by $CD8^+$ T cells in a short-term (24h) coculture setting *in vitro* [36]. As elucidated in chapter 5, inhibiting glycolysis could promote mitochondrial metabolism in $CD8^+$ T cells. In addition, this increases the formation of memory $CD8^+$ T cells and enhances their cytokine production and anti-tumor function [48]. However, the effect of long-term use of glycolysis inhibitors on tumor infiltrating immune cells must be confirmed in additional studies.

The encouraging pre-clinical evidences of combining metabolic targets with immunotherapy have recently manifested into early-stage clinical trials in melanoma. Of note, metformin is being tested in combination with anti-PD-1 in two independent

trials in patients with unresectable stage III and stage IV melanoma (NCT03311308; NCT04114136). If the combinations are deemed tolerable and efficacious in these early trials, this would reinvigorate the interest in targeting tumor metabolism and pave the way for a new line of combination approach to immunotherapy.

Patient stratification for personalized medicine – lessons from melanoma

We have explored the use of IFN-γ score determined using the baseline biopsies of patients with resectable stage III melanoma, to predict pathological responses and thereafter escalate or de-escalate neoadjuvant treatment based on IFN-γ low versus IFN-γ high scores, respectively (chapter 3). This approach allowed the de-escalation of treatment (use of anti-PD-1 monotherapy instead of anti-PD-1+anti-CTLA-4) in the patients with a high IFN-γ score in their baseline tumor biopsies. Most importantly, the DONIMI trial prospectively confirmed the data of OpACIN-Neo study wherein, a high IFN-γ score could predict pathological response to ICB in the neoadjuvant setting in melanoma [9]. Using such predictive biomarkers will help in identifying the patient sub-groups that are in need of combination or alternatives to ICB, allowing personalization of therapy. This also mitigates the risk of exposing patients who can benefit from ICB alone, to potential toxicities that might be associated with novel combination therapies.

Predictive biomarkers to ICB should be evaluated and optimized for individual tumor types. Tumor mutational burden (TMB) is known to faithfully predict response to ICB only in a small subset of cancer types, including melanoma [49]. The lack of predictive value in other tumor types is mainly ascribed to an absent correlation between $CD8^+$ T cell infiltration and the presence of neoantigens in these cancer types [49]. On the other hand, it is plausible that certain biomarkers are shared across tumor types. For example, IFN-γ score can predict objective responses to anti-PD-1 therapy in head and neck squamous cell carcinoma and gastric cancer as well [5]. Confirming this in larger cohorts can help in evaluating the utility of this signature across different cancer types. Moreover, T cell infiltration, measured as T cell-inflamed gene expression in the tumor [5], is positively associated with response to anti-PD-1 across tumor types [50]. Using gene expression profiles determining $CD8^+$ T cell infiltration and activity as a predictive tool could be a starting point to stratify patients into sub-groups with different likelihood of responses, especially in trials evaluating combination approaches to ICB.

Conclusion

ICB therapy has resulted in an unprecedented increase in overall survival in patients with unresectable stage III and IV melanoma [51]. Moreover, treating high risk resectable stage III melanoma patients with ICB in the neoadjuvant setting has significantly increased the response rates and prolonged relapse-free survival [52]. Results from the ongoing phase III NADINA trial (NCT04949113) could confirm the results from the recent SWOG S1801 trial [53], showing increased benefit of neoadjuvant PD-1 blockade compared with adjuvant treatment, making the use of this approach as standard-of-care. Research efforts over the recent years has also enabled the prospective identification of the non-responding patient sub-group by the use of baseline biomarkers such as low IFN-γ response signature in tumor biopsies. This thesis describes approaches to increase anti-tumor $CD8^+$ T cell responses and reduce suppression by Tregs with an aim to enhance response of a sub-group of patients with advanced stage melanoma to ICB. Moving forward, targeting metabolic pathways could be explored for their therapeutic benefit upon combination with ICB. It is however important to note that novel combination approaches to immunotherapy, especially in the neoadjuvant setting, might result in increased toxicities. Evaluating safety profiles of drug combinations in early development stages is therefore essential. The use of other checkpoint blockade molecule combinations in the neoadjuvant setting, such as lymphocyte activation gene 3 (LAG3) + PD-1 blockade is associated with reduced irAEs [54], compared with anti-PD-1+anti-CTLA-4. Such alternative combinations could be explored to obtain increased response rates without overt toxicities. We have come a long way in the treatment of advanced stage melanoma from palliative care-only to immunotherapies leading to durable responses. The next step of tackling lack of response of a sub-group of patients by the use of alternative approaches no longer seems like an unattainable feat.

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