Improving response and reducing toxicity to immune checkpoint blockade therapy in melanoma

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General introduction and outline of the thesis

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The majority of patients with early-stage melanoma benefits from surgical resection. However, for patients who develop advanced disease, characterized by metastasis, which can be either lymphogenic (stage III) or distant (stage IV), the treatment options have long been limited. Until 2011, the mainstay of treatment for patients with unresectable metastatic melanoma was the dacarbazine, which was administered as single-agent chemotherapy. It showed responses in only 5% to 20% of patients, resulting in poor long-term clinical outcome (1). Other therapeutic options included high dose interleukin-2 (IL-2) or radiotherapy, however, these therapies also achieved minimal clinical benefit for these patients (2, 3, 4). In recent years, major successes concerning treatment of advanced melanoma have been made by the introduction of BRAF inhibitors and ICB therapy, shifting the paradigm from once considered an incurable disease to a potential treatable disease with a curative rather than a palliative intent.

Melanoma is characterized by a high mutational load and tumor heterogeneity, making it difficult to effectively be targeted by true driver mutations. The most frequent driver somatic mutations to be identified are activating mutations of BRAF, occurring in 40%-60% of the cutaneous melanoma patients. This mutation occurs most commonly at amino acid position 600 at which normal valine is substituted, in nearly all cases, by glutamic acid ($BRAF^{V600E}$), or, less common, by lysine ($BRAF^{V600K}$). These mutations in BRAF results in a constitutive activation of its downstream signaling through the mitogen-activated protein kinase (MAPK) pathway, resulting in increased proliferation (5, 6, 7). Targeting BRAF with the ATP-competitive inhibitors vemurafenib or dabrafenib, thereby preventing constitutive activation of the MAPK pathway, proved to be a major advance in the treatment of patients with advanced melanoma, prolonging overall survival (OS) and progression-free survival (PFS) (8, 9). Combining BRAF inhibition with the MAPK kinase (MEK) inhibitor trametinib demonstrated to be even more effective. In spite of these advances, the responses to MAPK pathway inhibition were not long lasting and resistance to these treatment regimens arises (10, 11, 12, 13, 14). Therefore, efforts to improve durability in response is one of the important challenges to face to improve treatment of advanced melanoma.

The use of ICB therapy, too, has revolutionized the treatment of advanced melanoma, as well as for other malignancies, such as Hodgkin lymphoma, Merkel cell carcinoma, renal cell carcinoma, bladder cancer, microsatellite instability-high tumors, and non-small cell lung carcinoma (15, 16, 17, 18, 19). The use of ICB therapy is based on the ability of cancer cells to evade the immune system. Immune checkpoints are expressed by healthy host cells and play an important role in the maintenance of immune self-tolerance, preventing autoimmunity to occur. However, also tumor cells are capable of inducing the expression of these immune checkpoints, thereby inhibiting the attack by immune cells, thus causing immune evasion. The use of ICB therapy blocks these

checkpoint molecule interactions to release the brake on the immune system (20, 21). The molecules targeting cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed cell death 1 (PD-1) are currently the clinically most relevant checkpoints, regulating the immune responses at different levels and by diverse mechanisms.

Inhibition of the CTLA-4 checkpoint was the first treatment to improve OS in patients with advanced melanoma (22). CTLA-4 is a co-inhibitory receptor, expressed by both $CD4^+$ and $CD8^+$ T cells. Upon T cell receptor (TCR) recognition of an antigenic peptide within the major histocompatibility complex (MHC) on the surface of an antigen presenting cell (APC), a second signal is required for full T cell activation. This stimulatory signal can be either of stimulatory or inhibitory nature. In the case of co-stimulation, CD28 expressed by T cells interact with the ligand B7-1/B7-2 (also known as CD80 and CD86, respectively) on APCs, which results in the activation of T cells. However, when T cells expresses the co-inhibitory molecule CTLA-4, which has higher affinity for B7, and thus outcompetes CD28's positive co-stimulatory signal, T cell activation is inhibited. Monoclonal antibodies that block CTLA-4 prevent this interaction, thus allowing CD28 to bind to its ligand, leading to T cell activation (23, 24, 25). Another postulated action mode of anti-CTLA-4 antibodies is to affect regulatory T cells, although the exact mechanism is not known (26, 27, 28). In 2011, the first anti-CTLA-4 treatment, ipilimumab, was approved, showing an improved survival in patients with metastatic melanoma (29).

In contrast to CTLA-4, the major role of the immune checkpoint PD-1 is to limit the activity of T cells in the periphery at the time of an inflammatory response, thereby preventing potential autoimmunity. Upon activation, PD-1 is expressed on T cells, as well as other immune cells, such as B cells and natural killer (NK) cells. When PD-1 engages to one of its ligands, programmed death-ligand (PD-L) 1 and 2, TCR signaling is inhibited, limiting cytokine production, proliferation and possible lytic activity (30). PD-L1 and PD-L2 can be expressed also by tumor cells, thereby preventing immune attack (31). This interaction is blocked by anti-PD-1 (and anti-PD-L1) antibodies. The first phase III clinical trials (KEYNOTE-006 and CheckMate 067), testing the anti-PD-1 antibodies pembrolizumab and nivolumab, showed both a higher response rate, 46% and 45% respectively, and a superior efficacy compared with ipilimumab, with a 5-year OS of 43% for pembrolizumab and 44% for nivolumab (32, 33, 34). As the two checkpoint inhibitors have non-redundant roles in regulating roles in immunity, the co-inhibition of CTLA-4 and PD-1 was initiation in an effort to further improve this response rate. The combination of ipilimumab and nivolumab showed to be more effective compared to ipilimumab monotherapy, showing a response rate of 58%, with a 5-year landmark OS of 52% (32).

Given that ICB blocks the immune checkpoints involved in self-tolerance and limiting autoimmunity, it is not surprising that ICB induces adverse events (AE) that mimic autoimmune disease. Especially in the setting of combination therapy, a high rate of severe immune related (ir)AEs are observed, in which up to 55% of patients treated with ipilimumab and nivolumab develop grade 3 or 4 AEs (16, 35, 36, 37, 38, 39, 40, 41). These irAEs develop usually within the first 3 months of treatment and require prompt management (e.g., steroids, anti-tumor necrosis factor-alpha (TNF-α) antibody). The irAEs can affect any organ, most commonly the skin, gastrointestinal, endocrine system and liver (42). While these irAEs can be reversed in the majority of cases, some cause permanent damage, e.g., endocrinopathies, and can even be life threatening, e.g., causing polyradiculitis, myocarditis or Addison crisis.

In spite of the significant improvements offered by ICB in advanced melanoma patients, a substantial proportion of late-stage patients fail to respond, which can be caused either by primary or acquired resistance. For primary (or innate) resistance, no clinical response to treatment is observed, while for acquired resistance an initial response is obtained, but disease progression follows. Although it is largely unclear what mechanistically determines response and resistance, some predictors have been established in the recent years. Different factors within the tumor microenvironment (TME) influence the therapeutic efficacy ICB, including infiltration of $CD8^+$ T cells, PD-1 expression on T cells, interferon-gamma (IFN-γ) receptor pathway, 2,3-dioxygenase (IDO) activity, presence of regulatory T cell and myeloid-derived suppressor cells. In addition, tumor intrinsic factors impact also ICB response, including tumor mutational burden (TMB), alterations in the MHC expression pathway and PD-L1 expression (43, 44, 45, 46).

Considerable knowledge has been gained on the resistant mechanism involved in the therapeutic activity of ICB treatment. However, still a substantial percentage of patients does not respond (durable) to ICB treatment and many question remain. Therefore, in this thesis, I aim to improve our understanding of ICB efficacy and increase the proportion of patients who durably respond to these therapies (chapter 2-6). In the second part of the thesis, I also aim to find causes underlying AEs (chapter 7-8).

While the use of ICB for the adjuvant treatment of patients with surgically resectable melanoma has demonstrated to improve recurrence-free survival (RFS), the effective scheduling of surgery and immunotherapy and its duration are not well elucidated. Preclinical studies suggest that surgery followed by adjuvant therapy might be suboptimal as compared with an approach in which immunotherapy is applied before surgery (neoadjuvant ICB). In **chapter 2**, we focus on the promise of neoadjuvant ICB therapy and discuss the existing rationale for the use of neoadjuvant immunotherapy, its apparent strengths and weaknesses, and implications for design of future clinical

trials. In **chapter 3**, we show that neoadjuvant ipilimumab plus nivolumab treatment in stage III melanoma patients (OpACIN(-neo) studies) induces durable responses. Biomarker analyses revealed that patients with a high TMB and a high IFN-γ gene expression signature score were associated with higher responses and lower risk of relapse. In addition, we show in **chapter 4** that patients with a low tumor infiltration of conventional type 1 dendritic cell (cDC1), a DC subtype that excels in cross-presentation, were less likely to respond. This patient population could potentially benefit from therapies that enhance cross-presentation of tumor antigens, which led us to develop a cross-presentation assay to screen for over 5,500 compounds to find enhancers of this process. The screen identified 145 compounds, including AZD5582, an antagonist of inhibitor of apoptosis proteins (IAPs) cIAP1, cIAP2 and XIAP. We demonstrate that AZD5582 induces enhanced antigen import from endolysosomes into the cytosol, increased DC maturation and activation and a reduction of tumor growth *in vivo*.

In contrast to cutaneous melanoma (CM), the response rate upon ICB in uveal melanoma (UM) are disappointing (ranging from 0-15%), and none of the conducted phase III clinical trials has reported significant OS benefit. In **chapter 5**, we characterize liver metastasis from patients with UM and CM, in order to dissect the potential underlying mechanism in differential response upon ICB. While TMB was different between CM and UM metastases, tumor immune infiltration was similar. Only a higher ratio of exhausted CD8 T cells to cytotoxic T cells, as well as lower expression of PD-L1, was observed in UM liver metastases compared to CM metastases.

The response rates upon ICB in stage III melanoma are higher compared to stage IV disease. Given that successful ICB depends on systemic immune response, we hypothesized that systemic immune suppression might be a mechanism responsible for lower response rates in late-stage disease, and potentially also with disease recurrence in early-stage disease. Accordingly, in **chapter 6**, we analyzed the systemic protein expression of different cohorts of melanoma patients with mass-spectrometry based protein profiling and Olink analysis using an unbiased approach. We found leucine-rich alpha-2-glycoprotein 1 (LRG1) to be associated with worse prognosis, especially non-responder patients to neoadjuvant ICB (OpACIN-neo study) with high LRG1 expression had poor prognosis, which was validated in an independent cohort (PRADO study).

The similarity of irAEs to autoimmune disorders fuels the hypothesis that AEs may be linked to susceptible genetic loci related to various autoimmune diseases. **Chapter 7** of this thesis discusses the potential susceptible loci that might be associated with ICB-induced AE. Incorporation of new predictive biomarkers that could exclude poor candidates (patients who are not responding and have high change of severe toxicities) for novel therapeutic modality would be of high value. In **chapter 8**, we investigate

whether previous infection with neurotropic bacteria and viruses predispose patients to neurotoxicity upon ICB treatment.

In **chapter 9**, I summarize the research presented in this thesis, and pose a number of points I deem relevant for future directions to improve outcome for ICB treated melanoma patients.

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