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

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General discussion and perspective

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Rational for why neoadjuvant is more effective than adjuvant ICB

ICB therapy has demonstrated efficacy in a proportion of patients with irresectable melanoma (1). More recently, in an effort to improve outcome for patients with surgically resectable melanoma, adjuvant ICB after surgery is administered with the aim to eliminate microscopic or residual disease, and consequently preventing relapse. The use of adjuvant therapy of anti-CTLA-4 and anti-PD-1 after surgery indeed improved recurrence-free survival (RFS) and overall survival (OS) in melanoma patients with high risk of relapse (2, 3, 4), improving 3-year RFS from 34.8% (placebo group) to 46.5% (ipilimumab group) (2). Although adjuvant ICB in resectable melanoma is promising, surgery followed by adjuvant therapy might be a suboptimal scheduling of therapy as compared with the approach of applying ICB prior to surgery (neoadjuvant immunotherapy). There are several advantages in applying ICB therapy prior to surgery instead of after surgery. As also outlined in **chapter 2**, neoadjuvant therapy allows one to determine ICB treatment response, reduces tumor burden prior to surgery and to use pathologic response data as surrogate biomarker for RFS and OS. Moreover, neoadjuvant ICB therapy enhances T cell activation while it is still exposed to antigens, since the major tumor mass (including infiltrated T cells) is still present. This can lead to reinvigoration of pre-existing tumor-specific $CD8^+$ T cells, as well as the activation of new tumor-specific $CD8^+$ T cell clones. This could be the result of tumor antigen release after the activation of the existing tumor-specific T cell response, which in turn can be presented by antigen-presenting cells (APCs), thereby priming naïve T cells with tumor specificity for these antigens. The phenomime of re-expansion was shown for the first time in the phase 1b OpACIN study (NCT02437279), comparing neoadjuvant versus adjuvant ipilimumab plus nivolumab in macroscopic stage III melanoma patients (5). Patients who received neoadjuvant ICB therapy had a superior expansion of T cell clones that were detectable at baseline as well as newly detected T cell clones compared to adjuvant-treated patients, proving the concept of better T cell expansion. It is of interest to see whether this will also lead to improved protection against tumor reemergence. Patients who were not capable of broadening their detectable T cell repertoire after neoadjuvant ICB, had a relapse (5). An interesting observation has also been made in mouse models following neoadjuvant immunotherapy, showing persistence of tumor-specific $CD8^+$ T cells with an effector memory or central memory phenotype in the blood of tumor-free mice throughout life (6).

High response rates are observed in patients treated with neoadjuvant ICB. The phase 2 OpACIN-neo study (NCT02977052), treating macroscopic stage III melanoma patients with different dosing of neoadjuvant ipilimumab plus nivolumab, showed high response rates of 65-80% (depending on treatment arm) (7). As we show in **chapter 3**, this is translated into a remarkable relapse free survival, showing an estimated 2-year RFS of

84%. Especially patients with a pathologic response had a good prognosis, having a 2-year RFS of 97% versus 36% for patients without a pathologic response. Also the PRADO extension cohort of the OpACIN-neo study showed a high pathologic response rate (72%, including 61% major pathologic responses). The 2-year RFS was 93% for patients with major pathologic response, 64% for patients with a pathologic partial response (pPR) and 71% for patients with a pathologic non-response (pNR) (8). In the pooled analysis of the International Neoadjuvant Melanoma Consortium (INMC), in the group of patients with a pathological complete response (pCR), near pCR or partial pathological response very few relapses were seen (2-year RFS 96%) and at moment of publication no patient died from melanoma (9). This pooled analysis included 141 patients from four neoadjuvant clinical trials (5, 7, 10, 11). This high RFS is persisted, as the longer follow-up time of the OpACIN-neo study showed an estimated 3-year RFS and OS rate of 83% and 92% respectively, with an estimated 3-year RFS rate of 95% for patient with a pathologic response versus 37% for patients without pathologic response (12).

While this data from current neoadjuvant clinical trials is promising, the efficacy of neoadjuvant versus adjuvant therapy needs to be confirmed in a phase 3 trial. The OpACIN study was the first study comparing neoadjuvant versus adjuvant ICB treatment in a relatively small cohort of patients (n=20). As outlined in **chapter 3**, the 4-year event-free survival (EFS) and OS rate for the neoadjuvant arm was 80% and 90% versus 60% and 70% for the adjuvant arm. The phase 2 SWOG S1801 study (NCT03698019), treating patients with detectable and resectable stage IIIB-IV melanoma with either adjuvant or neoadjuvant pembrolizumab, showed after a median follow-up of 14.7 months a EFS rate of 72% for patients receiving the neoadjuvant treatment compared to 49% for patients receiving the adjuvant treatment. This is the first evidence proving that neoadjuvant ICB therapy is clinically superior to adjuvant ICB therapy. The efficacy of neoadjuvant ICB versus adjuvant therapy needs to be confirmed in the phase 3 NADINA trial (NCT04949113)(13), until neoadjuvant ICB therapy may become standard of care for patients with early-stage cancer that are at high risk of relapse.

Strategies to improve outcome non-responder patients

Although neoadjuvant ICB is promising, a proportion of patients is not responsive to ICB therapy. These patients without a pathological response have a poor RFS (2-year 36%; **chapter 3**). Therefore, it is particularly important to identify underlying mechanisms of resistance and biomarkers that predict response. Consequently, new therapeutic strategies that target these resistant mechanisms can be explored, potentially enhancing response to ICB therapy.

Our understanding of the mechanisms of ICB efficacy and resistance is continuously evolving and several discoveries of factors that impact response and resistance have been made in the recent years. These include tumor-intrinsic factors, such as tumor mutational burden (TMB) (14), antigen-presentation machinery status (15), interferon (IFN) signaling (16), Wnt/b-catenin pathway (17), programmed death-ligand 1^+ (PD-L1) extracellular vesicles (18), as well as the composition of the tumor microenvironment, including stromal components, $CD8^+$ T cells, myeloid-derived suppressor cells, tumor-associated macrophages and B cells (19). In addition, it became apparent that also other factors such as metabolic status (20), intratumoral microbes (21), gut microbiome (22) and host-extrinsic factors (19) play a role in the response to ICB therapy. In the setting of neoadjuvant therapy, as described in **chapter 3**, we observed that patients of the OpACIN-neo study with a tumor baseline low interferon-gamma-related gene expression signature score (IFN-γ score) had a worse prognosis. In addition, we identified that a tumor baseline low expression of the Batf3 dendritic cell (DC) associated RNA gene signature (Batf3-DC score), a DC subtype that excels in (tumor) antigen cross-presentation, was also associated with lower response rate (**chapter 4**), which confirms this finding of the OpACIN cohort (23). Combining either the IFN-γ score or Batf3-DC score with TMB, showed that patients who also had a low TMB were at highest risk for relapse, showing 2-year RFS of 50% for patients with a low IFN-γ score/low TMB and 2-year EFS 38% for patients with a low Batf3-DC score/low TMB. This subgroup of patients has poor prognosis and may benefit from additional therapies.

This approach is been tested in the DONIMI study (24), stratifying patients according to the tumor baseline IFN-γ score. Patients with a low IFN-γ score received the addition of domatinostat (a class I histone deacetylase inhibitor) to anti-PD-1 + anti-CTLA-4 treatment, with the rational to induce IFN-γ response score as is observed in preclinical (melanoma) models. Although the addition of domatinostat did not appear to increase treatment efficacy, this strategy of rationalized personal treatment is promising. Based on this idea, patients with a low Batf3-DC score might benefit from therapies that enhance cross-presentation of tumor antigens. Consequently, in **chapter 4** we conducted a repurposing compound screen, with the aim to identify compounds that improve T cell priming by cross-presentation of tumor antigens by DCs. We found AZD5582, an antagonist of inhibitor of apoptosis proteins (IAPs), to significantly enhance antigen cross-presentation, T cell proliferation and activation. We observed that AZD5582 treatment showed an additive effect to anti-PD-1 treatment *in vivo*. It remains uncertain how this translates to a human setting, since AZD5582 is still in the pre-clinical development stage. It would be of interest to test the potential of AZD5582 in patient-derived human explant systems, such as human patient-derived tumor fragment platform (25), and more specifically, in tumor fragments with a low Batf3-DC score to establish whether AZD5882 could induce a response signature in these patients.

A remaining challenge is that these patient-derived human explant systems usually lack viable DCs (or draining lymph node), and thereby limiting to establish the DC potential, which is key in our system.

Encouraging results have been observed for the first clinical trials enhancing DC function (NCT03084640 (26); (NCT01976585) (27)) and antigen presentation (NCT01970358 (28)). The combination of CpG-A TLR9 agonist (vidutolimod) and pembrolizumab resulted in durable responses in 25% of patients with advanced melanoma who had progressive disease or stable disease prior anti-PD-1 therapy (NCT03084640 (26)). Personalized neoantigen vaccination of patients with high-risk melanoma after surgery resulted in no recurrence at 25 months in four out of six patients (NCT01970358 (28)). Additional strategies for enhancing antigen cross-presentation which does not require an accessible lesion for intratumor administration of the agent(s) and costly prior identification of specific epitopes has an advantage. Hence, enhancing T cell proliferation after antigen cross-presentation using small molecules provides a promising strategy.

In **chapter 4**, a total of 145 different compounds were identified to significantly improve T cell proliferation after cross-presentation of tumor antigens by DCs. These compounds could in theory also have direct effect on T cell proliferation or tumor cells, bypassing DCs. For AZD5582, our top-hit, we established a direct effect on antigen cross-presentation and DC function. Although the aim of the screen was to target antigen cross-presentation, affecting T cell proliferation directly is not per se a bad addition. It would be of value to test if the other top hits enhance anti-tumor immunity. For future studies, I would propose to test these 145 hit compounds in different assays, including direct tumor toxicity, T cell cytotoxicity assay, T cell effector function (e.g. proliferation, cytokine release, activation markers) and regulatory T cell suppression assay. Using this strategy, a potential compound with different properties that influences anti-tumor immunity positively could be identified. This compound could shift the balance from a tumor suppressive to a tumor inflammatory environment on multiple levels, changing possibly the outcome for patients with poor prognosis.

Beyond stage III cutaneous melanoma: how to improve efficacy

Patients with UM, a rare subset of melanoma, have a very poor prognosis, especially patients who develop metastasis. Despite their shared origin (melanocytes), ICB therapy showed advances for late-stage cutaneous melanoma (CM) (29), but the response rates in UM are disappointing (ranging from 0% to 15%) (30, 31, 32). The profound cause for this disparity in response is unknown. To improve the understanding of resistance of UM to ICB therapy, we compare in **chapter 5** metastases of CM and UM patients

from the same metastatic site (liver) to avoid organ specific differences. The liver has been shown to be the least responsive metastatic site to ICB therapy (33). When characterizing liver metastasis of CM and UM, a higher TMB, and hence a higher predicted neoantigen load was found for CM compared to UM. However, the expression of melanoma differentiation antigens (MDA) (PMEL/gp100, MelanA/MART-1, tyrosinase) was high or even higher for UM patients than CM patients. These MDA expressed by tumor cells also play a role in tumor recognition by endogenous self-antigen reactive T cells. Therapies that target these MDA (e.g. CAR-T cell or bispecific molecules) are therefore potential promising treatment options for UM. In 2020, at the time of writing chapter 5, we reported that none of the conducted phase 3 clinical trials reported significant OS benefit for metastatic UM patients. This changed with the introduction of tebentafusp (NCT0307392), a bispecific IMCgp100 antibody. Tebentafusp treatment significantly improved OS, showing a 1-year OS of 73% for the tebentafusp group and 59% in the control group (34). PFS was also significantly higher for patients treated with tebentafusp compared to the control group, 31% versus 19% respectively at 6 months. This bispecific molecule consists of an affinity-enhanced T cell receptor fused to an anti-CD3 effector that is reactive to gp100-positive cells. The data of this phase 3 is promising, but tebentafusp is at present restricted to patients positive for HLA-0201, which is 50% of the Caucasian population, and needs to be expanded in order to be a treatment option for a larger group of patients.

Despite the difference in TMB, the T cell infiltration of CM and UM liver metastases were comparable (**chapter 5**). Although UM is thought to be representing a tumor variant that is poorly recognized by the immune system, it was previously shown that these infiltrated T cells can recognize tumor antigens, as clonal T cell expansion (35) and tumor-infiltrating lymphocyte (TIL) reactivity to autologous tumors (36) is observed. This theory is further challenged by a phase 2 study of TIL therapy in metastatic UM patients (NCT01814046) (37), inducing objective tumor regression in seven (35%) of 20 patients. Moreover, the expanded TILs showed strong anti-tumor reactivity in 50-60% of cases. This indicate that the resistance to immunotherapy by UM tumors cannot be explained by a lack of immune infiltration and reactivity. Alternatively, it could be that these T cells are dysfunctional/exhausted, explaining the lack of efficacy. We observe a higher ratio of exhausted CD8 T cells to Th1, cytotoxic and CD8 T cells (**chapter 5**). As these exhausted T cells are defined by LAG-3 expression, UM patients might benefit from anti-LAG-3 therapy, particularly since tumor infiltrating immune cells express pre-dominantly LAG-3, rather than PD-1 or CTLA-4 (35). The first clinical trial testing nivolumab plus relatlimab (anti-LAG-3) in patients with metastatic UM is currently ongoing (NCT04552223) and it needs to be elucidated if anti-LAG-3 therapy is indeed a potential candidate to improve immunotherapy for UM patients. Another approach to improve response of UM liver metastasis is directed to enhance antigen presentation

and increase immunomodulatory effect by combining ICB with locoregional therapy, which has shown to be effective for liver metastasis. This is currently tested in the CHOPIN trial (NCT04283890), were ICB is combined with percutaneous hepatic perfusion with melphalan (38).

The advent of ICB therapy has improved the prognosis for stage IV CM, however, this is a patient group with poor prognosis. A lower response rate to ICB is observed for patients with stage IV disease as compared to stage III disease (39, 40, 41, 42, 43). This patient group would in particular benefit from strategies that improve treatment efficacy. These patients have potentially a higher degree of systemic immune suppression, since also a lower frequency of high grade irAEs is observed at similar dosing and number of courses (43, 44). Based on this hypothesis of systemic immune suppression, we analyzed in **chapter 6** serum/plasma of melanoma patients to identify systemic biomarkers that are associated with disease progression and recurrence. We identified lactate dehydrogenase (LDH), C-reactive protein (CRP), serum amyloid A (SAA), IL-8 and IL-10 to be associated with disease progression in our cohort, confirming previous findings (45, 46, 47, 48, 49, 50). In addition, we found leucine-rich alpha-2 glycoprotein 1 (LRG1) to be higher expressed by patients with progressive disease, which was also found to be higher expressed in patients without a response to neoadjuvant ICB treatment that developed disease recurrence. These proteins could serve as markers for intensified adjuvant treatment and follow-up. While there is an indication that these markers contribute to immune suppression, it would be of interest to establish if these proteins are indeed causal for hampered immune response. I would propose to translate these findings back to the lab and assess protein neutralization in (neo)adjuvant mouse models. LRG1 has been shown to promote epithelial-to-mesenchymal transition (EMT) (51), dysfunctional angiogenesis (52) and modifies the TGF-b signaling pathway (53). The first results of anti-LRG1 are promising, showing reduced tumor growth, synergistic effect with anti-PD1 and improved vascular function, improving possibly the delivery of immunotherapy (52, 54, 55). In addition, we need to gain more mechanistic insight on the (potential) suppressive mode of action of these proteins. We could also learn from ongoing clinical trials, such as the study (NCT03400332) testing nivolumab + anti-IL-8 therapy (HuMax-IL8, BMS-986253) in patients with increased IL-8 serum levels (56). Together, these strategies will elucidate if these proteins are indeed possible targets for combination therapy with ICB.

An alternative treatment strategy for patients with stage IV disease that showed promising results is adoptive cell therapy with TILs. In a phase 3 trial (NCT02278887), comparing ipilimumab to TIL therapy in patients with unresectable stage IIIC or IV melanoma, showed an objective response rate of 49% in the TIL group and 21% in the ipilimumab group, with a median OS of 25.8 months in the TIL group and 18.9 months

in the ipilimumab group (57). Future studies should further evaluate which patients benefit from TIL therapy or alternatively ICB treatment in combination with rationalized combinations.

Toxicity to ICB: potential mechanisms and risk factors

At this time, we lack in depth understanding of the mechanisms underlying the development of irAEs. This is problematic, since the use of ICB for treatment of different cancer subtypes is rapidly increases and consequently the incidence of these irAEs will continue to rise. Moreover, ICB therapy moves towards adjuvant and neoadjuvant approaches in stage III disease with a curative intent, which makes reduction in (severe) irAEs even more important, since some patients may be cured by surgery alone. Therefore, efforts should be made to elucidate mechanisms of irAEs and to develop biomarkers to identify patients at highest risk.

The identification of potential risk factors is especially desired for irAEs that cause permanent damage and can be life threatening, which include neuropathies, cardiomyopathies, nephritis and endocrinopathies. Severe irAEs are more frequently observed in combination therapy of ipilimumab/nivolumab compared to monotherapy (58, 59, 60), and therefore, patients with a high change on such irAEs might benefit from alternative treatment schedules or drugs (e.g. targeted immunotherapeutic such as bi- and tri-specific antibodies (61)) to reduce toxicity. As outlined in **chapter 3** and **chapter 4**, patients with either a high tumor IFN-γ score or high tumor Batf3-DC score in combination with a high TMB have high response rate to ICB therapy, respectively 100% and 94%. Especially patients within these groups with a high risk for severe irAEs would potentially already benefit from monotherapy, and thereby, de-escalating treatment and skewing risk-benefit ratio to a more favorable balance. Patient stratification according to the IFN-γ score has been tested in the DONIMI study (24), treating IFN-γ signature high patients only with monotreatment of nivolumab. Such a personalized approach with the inclusion of risk factors for serious irAEs would potentially decrease the incidence of these irAEs.

Until now, there are no potential biomarkers that predict the development and severity of irAEs which can guide treatment decision making. The similarity of irAEs to autoimmune diseases argues for a possible link for a shared genetic pre-disposition. In **chapter 7**, we discuss which susceptible loci are associated with various autoimmune diseases and are potentially relevant for ICB treatment-induced irAEs. These suggested susceptible loci could be the basis for large association studies in ICB treated patients. Indeed, certain HLA alleles that are known to predispose to autoimmune disease have been associated with various irAEs. The described HLA types predispose patients during

ICB for pruritis, colitis (62) and arthritis (63), and seem to be disease specific so far. The development of high-throughput sequencing technologies allows large genome wide association studies in ICB treated patients. A challenge for this approach is that it would require a large number of patients with both low- and high-grade toxicity and appropriate controls, including patients treated with ICB without any irAEs as well as healthy individuals. A multi-national collaborative effort is needed for gathering enough patient samples to identify susceptible loci with confidence, especially for rarer irAE subtypes.

Besides genetically pre-disposition for irAEs, several mechanisms have been proposed that contribute to development of irAEs in patients after ICB therapy, which do not need to be mutually exclusive. Autoreactive T and B cells to healthy tissue are thought to be key components in the development of irAEs. These autoreactive immune cells could either be pre-existing or *de novo* generated by tumor cell death and release of additional (potentially self) antigen (epitope spreading) (19, 64, 65, 66). This autoreactive immune reaction could turn from quiescent to active by alteration of local/systemic cytokine profiles. In **chapter 8**, we propose that patients who develop neurologic irAEs after ICB therapy were previously exposed to infections with neurologic mimicry, resulting in an autoreactive (re-)activation to shared self-antigens from the nervous system. This immunological cross-reactivity of an immune response to an environmental agent (e.g. infectious agent or vaccine) cross-reacting with self-antigens from the nervous system is associated with development of autoimmune neuropathies (67, 68). In our study we fail to show an association between previous neurotropic infections and development of neurologic irAEs. However, there are there are some limitations to this study in order to be fully conclusive about this. Therefore, I propose to analyze the presence of antibodies and T cell clones directed against previously reported immunodominant epitopes (65, 68) (pre- and post-neurologic symptom) in a larger cohort and a more homogenous patient population, with higher number of patients with similar neurologic irAEs. This analysis will give more definitive results whether immunological cross-reactivity is (one of) the underlying cause of neurotoxicity after ICB treatment.

Concluding remarks and future perspectives

In the past decade, ICB therapy has improved treatment outcome for melanoma patients. The results of the first neoadjuvant ICB studies show higher (durable) response rates, and applying neoadjuvant treatment as standard of care will increase the benefit of ICB treatment to a larger group of patients. Nevertheless, there is a need to expand ICB therapy efficacy to a larger group of patients. Extensive efforts have been made to elucidate response and resistant mechanisms. Future studies are warranted to further understand whether these resistant mechanisms can be targeted and

form the basis of novel therapeutic strategies. It is of importance to identify patients upfront with unfavorable prognosis, since these patients potentially benefit from these novel combination treatment strategies. There should be a rationale behind these novel combinations, which need to be driven by pre-clinical analysis with laboratory interrogation. Moreover, identification of patients upfront that are very likely to respond to ICB therapy is also key, as these patients might benefit from ICB monotherapy and de-escalating treatment could reduce chance of (severe) treatment related toxicities. This strategy will expand the benefits of ICB therapy to a larger population of melanoma patients in a rationale and personal manner.

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