# Exploring and modulating the tumor immune microenvironment: Towards improving patient outcomes of immunotherapy in lung cancer

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## SUMMARY

Since long, the interaction between the host immune system and tumor growth/control has been of interest within the oncology research field. Unraveling the tumor immune microenvironment has led to significant new insights. One of the most studied tumor immune escape mechanisms is mediated through the inhibitory programmed death-ligand 1 (PD-L1)/programmed death 1 (PD-1) pathway. Binding of tumor-expressed PD-L1 to the PD-1 receptor on cytotoxic T cells activates an inhibitory signal leading to apoptosis or inactivation of the immune cells and thereby allowing the tumor to evade the host immune response. Development of immune checkpoint inhibitors (ICIs), e.g. PD-1/PD-L1 monoclonal antibodies, has led to long-lasting anti-tumor immune responses in patients with metastatic non-small cell lung carcinoma (NSCLC).

Overall response rates (ORR) and other patient outcomes are associated with the protein expression level of PD-L1 on tumors as assessed by immunohistochemistry (IHC) and is therefore widely used as a predictive clinical biomarker for response on PD-1/PD-L1 blockade. However, patients with PD-L1 negative tumors still have an 8% chance of response and even at the highest expression level, i.e. ≥ 50%, a 70% failure rate occurs. These outcomes show that the treatment option immuno-monotherapy as well as the biomarker PD-L1 expression both remain far from optimal.

This thesis sought to obtain a better understanding of the composition of the immune microenvironment and its interaction with NSCLC. Also, improvement in NSCLC patient outcomes was aspired by combining ICIs with a potential immune modulator: radiation therapy (RT). In **chapter 1**, a general introduction on the epidemiology, treatment options and possible biomarkers for immunotherapy in NSCLC are presented. Furthermore, a short description of the abscopal effect of RT, the out-of-field or systemic antitumor response after local radiation, and the biological rationale of augmented immune responses by combining RT with ICIs are given.

### PART I. Exploring the tumor immune microenvironment

To enable exploration of the tumor immune environment in NSCLC, we built a database of over 600 tumor samples from resected patients and collected patient' and tumor characteristics. IHC staining of PD-L1 expression on tumor cells (TC) and tumor-infiltrating immune cells (IC) separately and of CD8 infiltration, mutational data, and gene expression mainly of genes related to immunologic function were obtained.

In **chapter 2**, we sought to improve insights in the associations of PD-L1 expression and specific patient' or tumor characteristics. Only for PD-L1 positive TC associations were found (*KRAS* mutations and smoking), but none were found for PD-L1 expression on IC. We then explored overlap and differences between expression of PD-L1 on TC and IC. Our interest was raised by the fact that more than half of the cohort had a PD-L1 positive immune infiltrate, which was associated with other immune gene markers, but without upregulation of PD-L1 on TC. In a subsequent analysis, we found that in the subgroup of TC0/IC3 samples an impairment of IFNγ response in the TC might be responsible for the lack of upregulation of PD-L1 in these tumors. These findings may contribute to the understanding why

patients with PD-L1 TC negative NSCLC may still (only) require immune checkpoint blockade to gain response and survival benefit.

In **chapter 3**, we investigated immune gene expression by NanoString using the same resection cohort of early-stage NSCLC. We found that in adenocarcinomas (AD) the level of overall inflammation as assessed by immune gene expression was significantly higher compared to squamous cell carcinomas (SCC). This seemed to be related to a higher infiltration rate of immune cells within the tumor bed of AD compared to SCC based on a significant difference in tumor cell percentage between both histologies, i.e. tumor cell percentage being higher in SCC. This may suggest a different interaction of immune cells and tumor cells between the different histologies. Interestingly, a cluster of 34 genes, identified by unsupervised clustering, did not correlate with the general level of inflammation, the level of PD-L1 expression or $CD8^+$ T cell infiltration. Expression of this 34-gene cluster did not differ between AD and SCC histology, but high expression of this signature showed a clear OS benefit in SCC, but not in AD. This finding was validated in two independent NSCLC cohorts. We then tried to allocate the nature of this 34-gene signature and found the strongest correlation with Natural Killer (NK) cell related gene expression. Cell surface genes involved in NK cell recognition and killing - *ULBP2* and *HLA-C* – were significantly different between SCC and AD histology in favor of our hypothesis, namely that SCC may be more susceptible to NK cell killing than AD. Unfortunately, there is no established gold standard for assessing NK cell infiltration and/or activation level in tumor samples. Also, these cells are generally scarce within the tumor microenvironment and our IHC NK cell double-staining was not able to differentiate the 34-gene high from the 34-gene low samples. Almost all genes within our 34-gene signature could not be adequately measured by RNA sequencing techniques due to low expression levels of the genes in the signature. The further investigation of the biological rationale of such low-level signatures could bring new insights in the role of immune cells that are present within the tumor infiltrate in low quantities, like NK cells.

**PART II. Modulating the tumor immune microenvironment**

Although the improvements of patient outcomes by the introduction of PD-1/PD-L1-antibodies in advanced NSCLC have been impressive, there is still an urgent need for further investigation, especially for those patients not responding to immune checkpoint blockade. RT could be a potent modulator of the tumor microenvironment and could augment the antitumor immune response when combined with immunotherapy.

In **chapter 4**, we provided a review about the off-target effects of RT, the so-called abscopal effect. We describe the biological rationale how RT may counteract the mechanisms of failure of immunotherapy. Also, an oversight of pre-clinical and clinical data supporting augmentation of abscopal events by RT when combined with immune checkpoint inhibition is presented.

To investigate this possible clinical impact of the abscopal phenomenon, the PEMBRO-RT trial was set up. In this multicenter study, patients with advanced NSCLC that had received at least one prior line of chemotherapy but were immunotherapy-naïve, were randomized between pembrolizumab treatment (control arm) vs pembrolizumab treatment within one week after three doses of 8 Gy (24Gy/3) on a

single tumor lesion (experimental arm). Stratification was based on smoking status: <10 pack years vs ≥10 pack years. The primary end point was ORR at 12 weeks from randomization according to Response Evaluation Criteria in Solid Tumors (RECIST). The results of the PEMBRO-RT trial are presented in **chapter 5**. The intention-to-treat (ITT) population consisted of 76 patients. Although the ORR at 12 weeks doubled in the experimental arm compared to the control arm, this difference was not statistically significant. The PD-L1 negative subgroup experienced a significant PFS and OS benefit in the experimental arm compared to the control arm, but no differences were seen in the overall ITT population regarding these outcomes. No increase in treatment-related toxicity was observed in the experimental arm. So, although an augmenting effect of RT on the response to PD-1 blockade in patients with metastatic NSCLC was observed, the study did not meet its primary end point of prespecified criteria for meaningful clinical benefit.

The MD Anderson Cancer Center (MDACC) was analyzing results from a similar randomized trial of pembrolizumab alone vs pembrolizumab in combination with RT (50Gy/4, stereotactic body radiotherapy (SBRT) or 45Gy/15, traditional RT). In **chapter 6**, we present the results of the pooled analysis of these two randomized trials. By exploring the possible abscopal effect in a larger cohort of advanced NSCLC patients, we found not only a significant improvement of abscopal response rate (ARR) in the experimental arm compared to the control arm, but also a significant PFS and OS benefit was observed in the patients treated with pembrolizumab and RT. Because RT regimen was not applied randomly, but rather based on trial variability and/or physicians' discretion, statistical comparison between RT regimens was not feasible. However, the 45Gy/15 subgroup showed an ARR similar to the control arm, where the other two RT regimens produced an ARR over two times as high. Exploration of the absolute lymphocyte count (ALC) showed a more pronounced ALC decline in the 45Gy/15 subgroup in comparison to both other RT regimens, which provides a hypothesis of a detrimental effect on immune response by traditional fractionation that may require further investigation.

**Future perspectives**

Finally, **chapter 7** provides a short summary of the recent developments in the systemic treatment of advanced NSCLC and a general discussion on the previously described findings in this thesis is given. Alternative predictive biomarkers for response to ICIs are currently under investigation, but although promising, as of yet none of these biomarkers have proven themselves more accessible and/or reliable compared to PD-L1 expression on tumor cells by IHC. Further research will be needed to improve (first-line) treatment selection in advanced NSCLC patients. Also, an oversight of ongoing translational research on the blood and tumor samples collected during the PEMBRO-RT trial is presented in this chapter. Hopefully, these will bring further insights in associations between tumor and/or patient characteristics and abscopal benefit and therefore guide us how to proceed clinical implementation of the abscopal phenomenon. Furthermore, endeavors implementing the use of immunotherapy within earlier stages of NSCLC are ongoing. In locally advanced stage III disease, where immunotherapy is combined with concurrent chemoradiation might provide opportunities to further explore the combination of RT with checkpoint inhibition. Also, neo-adjuvant treatment of immunotherapy in resectable disease will allow to investigate loco-regional pathological investigation as well as systemic immune responses

and may aid in biomarker development. And also applying neo-adjuvant RT in this setting may bring useful insights and biomarker assessment to better identify the abscopal effect in a clinical setting. These may assist us in optimization further research protocols investigating the possible advantage of adding RT to systemic treatment like immunotherapy in particular in NSCLC.