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

Theelen, W.S.M.E.

CHAPTER 1

General introduction and outline of the thesis

Epidemiology of lung cancer

In 2018, lung cancer was reported as the most common cancer with 2.09 million new cases globally by the World Health Organization. Also, lung cancer was the leading cause of cancer death with 1.76 million deaths that year [1]. In the Netherlands, the incidence of lung cancer is still on the rise. In 2018, approximately 8000 men and 6400 women received a diagnosis of lung cancer. For Dutch men this number has been relatively stable during the last three decades, but for Dutch women the incidence of lung cancer has risen from around 1300 new cases 30 years ago to break through the 6000-barrier for the first time [2]. Tobacco smoking has been strongly associated with the development of lung cancer [3].

In general, lung cancer can be divided into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). The latter accounts for approximately 15% of lung cancer patients. This thesis focusses on NSCLC only, which again can be roughly divided into adenocarcinoma (AD), squamous cell carcinoma (SCC) and large cell carcinoma not otherwise specified (LCC NOS). Approximately half of the NSCLC patients finds themselves diagnosed with an incurable metastatic stage on first presentation, i.e. stage IV, but also around 30% of patients who initially present themselves with curable disease will eventually develop metastases [4].

Treatment of metastatic NSCLC

Only a very small fraction of patients with metastatic NSCLC can possibly be cured by a more aggressive regimen of a combination of systemic and local treatment, like surgery or radiation therapy (RT). At initial presentation, these patients have few metastatic sites. This situation is referred to as oligometastatic setting and is considered as having a maximum of five metastatic lesions and three organs involved [5].

In general, treatment options in patients with metastatic NSCLC should be considered as palliative. For decades the only systemic treatment option that had shown scientific benefit in NSCLC was chemotherapy [6, 7]. The optimal chemotherapy regimen consists of a platinum doublet, where carboplatin or cisplatin is combined with preferably a third-generation cytostatic compound: a taxane, gemcitabine, pemetrexed or vinorelbine. The only comparison in regard to histology has been made for pemetrexed vs gemcitabine. SCC showed shorter overall survival (OS) with a pemetrexed-platinum combination compared to the gemcitabine-platinum combination, while the opposite effect was observed in non-squamous histology [8]. After progression on first line treatment, only second line mono-chemotherapy can be considered a beneficial treatment option. Local therapy like surgery and especially RT can be applied for palliative reasons on a specific symptomatic tumor site in metastatic disease.

The identification of oncogenic drivers like somatic point mutations or deletions (e.g. EGFR) and gene fusions (e.g. ALK) in NSCLC and the subsequent blockade with specific tyrosine kinase inhibitors has brought impressive tumor responses and prolongation of progression free survival (PFS) compared to chemotherapy [9, 10]. Unfortunately, these improvements in treatment options still only apply to a minority of the lung cancer patients, especially in the Western population, and mostly concern non-smokers.

The era of immunotherapy

More recently, research focusing on unraveling the tumor immune microenvironment has led to significant new insights [11]. Tumors express antigens that arise from mutations within the tumor DNA, the so called neoantigens, which can be recognized by host T cells as non-self. In lung cancer, these mutations are generally caused by smoking, making NSCLC one of the tumors with the highest tumor mutational burden (TMB) [12]. Unfortunately, the triggered immunologic response can generally not overcome progression of tumor growth nor the development of metastatic lesions. The mechanisms of the escape of host immunity by the immunosuppressive environment induced by cancer cells includes down-regulation of cell surface major histocompatibility complex (MHC) class I molecules, secretion of immunosuppressive factors, lack of T-cell co-stimulation, and expression of immune inhibitory pathways [13-16].

The most studied immune inhibitory ligand is the programmed death-ligand 1 (PD-L1). PD-L1 expression has been identified in a wide variety of solid tumors including breast, colon, ovarian, melanoma, bladder, liver, gliomas, thyroid, thymic epithelial, head and neck and lung [17]. Besides aberrant expression by tumors, PD-L1 is also mainly expressed by antigen presenting cells (APCs) and endothelial cells [18]. Its receptor, programmed death 1 (PD-1), is expressed on a variety of cells: T cells, B cells, natural killer T cells, activated monocytes, dendritic cells and even on tumor cells [17]. Binding of PD-L1 to the PD-1 receptor on 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.

The high TMB and subsequent presence of neoantigens would make NSCLC highly susceptible to T cell recognition and killing, but based on the high incidence of NSCLC an immune escape mechanism apparently appears to prevent tumor immune attack. Indeed, the development of immune checkpoint inhibitors (ICIs), PD-1/PD-L1 monoclonal antibodies, has led to long-lasting anti-tumor immune responses in patients with metastatic NSCLC [19]. In 2015, the PD-1 inhibitor nivolumab became the new standard of care (SoC) for metastatic NSCLC that had progressed on platinum-doublet chemotherapy [20, 21]. In the same year, registration followed for the PD-1 inhibitor pembrolizumab and the PD-L1 inhibitor atezolizumab for the same indication [22, 23]. All three compounds showed superior OS compared to docetaxel.

Biomarkers for immunotherapy

Overall response rates (ORR) and other patient outcomes were associated with the protein expression level of PD-L1 on tumors as assessed by immunohistochemistry (IHC). The ORR in PD-L1 negative tumors was approximately 8%, but increased to 30% in patients whose tumors expressed high PD-L1 expression defined by PD-L1 expression on 50% of the tumor cells or more. Nivolumab was beneficial irrespective of PD-L1 expression, especially when the more benign toxicity profile of immunotherapy vs. docetaxel is concerned. In the pembrolizumab trial, no patients with PD-L1 negative tumors were allowed to participate. The companion diagnostic tool for PD-L1 assessment in the atezolizumab study also measured PD-L1 expression on tumor-infiltrating immune cells. PD-L1 expression on tumor cells and on tumor-infiltrating immune cells are associated with one another, but both also have been reported to be independently associated with higher response rates on atezolizumab [24]. Still, at the time of

designing the protocol for the PEMBRO-RT trial described in this thesis, assessing PD-L1 expression was not yet readily accessible for clinical use. During that period, profound skepticism had risen about the value of PD-L1 as a useful predictive biomarker for response on ICIs, because PD-L1 negative patient still had an 8% chance of response and the subgroup of patients with the highest PD-L1 expression still a 70% chance of failure of immunotherapy. There are several issues concerning scoring of PD-L1 expression by IHC. Each PD-1/PD-L1 checkpoint blocker has its own PD-L1 assay as companion diagnostic. Cut-off levels are different for each of the assays and where most of them score PD-L1 expression on tumor cells only, the atezolizumab diagnostic tool gives a combined tumor and immune PD-L1 score. The latter assay does not align regarding tumor cell staining with the other assays that do not score PD-L1 on immune cells [25]. Besides different assays, tumor heterogeneity of PD-L1 expression may play a role. In metastatic NSCLC, diagnosis is mainly retrieved based on a small biopsy of the primary tumor or a metastatic site, but PD-L1 expression may not be evenly distributed across all lesions. Investigation of PD-L1 expression as a prognostic biomarker in early stage NSCLC has led to conflicting results and two meta-analyses concluded that no statistically significant association between PD-L1 expression and OS could be established [26, 27].

$CD8^+$ cytotoxic T cells are key players in immunoediting, the process by which tumor cells are eliminated due to antigen mediated killing [28]. Compared to PD-L1 expression, there is reasonable evidence that increased density of tumor infiltrating lymphocytes (TILs) is associated with improved prognosis in NSCLC [29, 30]. In melanoma, infiltration of $CD8^+$ T cells was associated with higher response rate on ICIs [31], but no compelling evidence of a similar association has been published in NSCLC. As to date, opposed to PD-L1 expression, the assessment of CD8 infiltration is not an established clinical biomarker for immunotherapy in NSCLC.

Also, TMB has proven to be a predictive biomarker for response to immunotherapy in NSCLC and height of TMB appeared to be irrespective of the level of PD-L1 expression [32]. Although useful, assessing TMB is still an elaborate effort and therefore not readily accessible for use in a clinical setting.

Exploring the tumor immune microenvironment by gene expression analysis

Previously, gene expression analysis has been used to find prognostic biomarkers especially for early stage NSCLC [33-35]. Also, they have proven to aid in pathological diagnosis of lung cancer [36]. Aside from the PD-1/PD-L1 axis and $CD8^+$ T cells, numerous other immunosuppressive and immunostimulatory mechanisms play a role in the tumor-immune interaction. Gene expression analysis allows us to perform comprehensive immunoprofiling of the tumor immune microenvironment and can assist in dissecting the different components of the immune infiltrate. As mentioned, presence of TILs has shown prognostic benefit in NSCLC probably through the immunostimulatory mechanism they represent [37, 38]. On the other hand, myeloid-derived suppressor cells (MDSCs) and T regulator (Treg) cells have an immunosuppressive effect on cytotoxic T cells and may therefore be associated with NSCLC progression [39]. By defining metagenes for specific immune cell populations based on transcriptomic data, like performed by the Microenvironment Cell Populations-counter (MCP-counter) method and validated by IHC, it becomes possible to evaluate the composition of the tumor immune infiltrate and maybe even allocate some of the established prognostic gene signatures [40]. Much is still

unknown about the optimal composition as well as the unfavorable aspects of the tumor immune infiltrate, let alone how to influence the composition for the NSCLC patients' benefit. Besides a better understanding of the role and ratio of all these components of the tumor immune infiltrate, the localization of these immune cells with respect to tumor cells determined by IHC –stromal and/or intraepithelial- may also contain valuable information on different mechanism of immune-tumor interaction [41, 42].

Abscopal effect of radiotherapy

Although many aspects of the tumor immune microenvironment still need to be unraveled, efforts beyond PD-1/PD-L1 blockade have already been explored as potential immunomodulators to provoke tumor responses in itself or as an enhancement to ICI. In the last decades, an increasing amount of evidence has been gathered proving that ionizing radiation may have potential immunoediting abilities. As mentioned before, RT is frequently used in the palliative treatment of metastatic NSCLC to reduce local symptoms like pain or hemoptysis. However, a rare phenomenon of an out-of-field antitumor effect of RT has been described. Patients who received palliative doses of RT on a specific tumor location showed tumor shrinkage of non-irradiated tumor lesions. This was mostly seen in melanoma patients, but also NSCLC cases have been described [43, 44].

This phenomenon is referred to as the abscopal effect; 'ab scopus' meaning away from the target. The biological rationale for this observation is sought in an antitumor response of the host immune system. When RT manages to induce immunogenic cell death of tumor cells, release of tumor antigens and production of pro-inflammatory mediators is induced [45]. APCs are thereby activated and a subsequent uptake of tumor antigens occurs [46]. These APCs migrate to tumor draining lymph nodes, where presentation of tumor antigens to T cells takes place. Recognition of antigens leads to an increased activation of tumor-specific T cells, which are able to generate an antitumor response within the previous irradiated lesion. Tumor-specific T cells reach the irradiated tumor through the circulation guided by activation of the "stimulator of interferon genes" (STING) signaling pathway through the pro-inflammatory mediators type I interferons in dendritic cells [47]. In general, non-irradiated lesions carry overlapping tumor antigens with the irradiated lesion and therefor recognition of out-of-field tumor lesions can also occur: the biological rationale for the abscopal effect [48, 49].

Combining radiotherapy with ICI

In theory, this seems like a promising systemic treatment, but in reality, only several case reports are known with 'spontaneous' out-of-field responses after local RT. However, this postulation of an abscopal effect makes RT an interesting modality in combination with other immunomodulating agents, like ICIs. In addition to a radiation induced inflammation of the tumor microenvironment and induction of tumor-specific T cell responses, tumor immune escape mechanisms could be tackled by this combination. Tumors can escape recognition by activated T cells through downregulation of MHC class I molecules, which can be found on all cells in the body besides erythrocytes but including tumor cells. These molecules are arbitrary in self-recognition by displaying peptides from normal cellular protein turnover, therefor T cells will not be triggered to attack. If cells present non-self-antigens, like tumor neoantigens, on their MHC molecules, T cells will proceed to cell killing on recognition. By downregulating their MHC

molecules and subsequent loss of neoantigen-presentation tumor cells are able to escape the immune system. RT has proven to upregulate MHC expression on tumor cells [50]. In addition, RT may promote a more pro-inflammatory tumor microenvironment by the elimination of immune suppressive cells. For example, RT has shown to be able to differentiate macrophages from an immune-inhibitory M2 towards an immune-stimulatory M1 phenotype [51]. Unfortunately, induction of immune-inhibitory cells by RT has been described as well, probably due to differences in tumor models or radiation regimens [52]. Interestingly, the pro-inflammatory induction of RT may in itself have detrimental effects on the tumor immune response through subsequent the upregulation of immune checkpoints, like PD-L1, causing tumor immune escape [53].

The described immunomodulating effects -especially the latter being a direct encouragement for this hypothesis- have led to exploring whether the combination of RT with immunotherapy would indeed lead to synergy in pre-clinical in vivo and in vitro solid tumor models [49, 54, 55]. Positive results have led to the development of clinical trials testing the safety and efficacy of this approach.

Outline of this thesis

This thesis sought to obtain a better understanding of the composition of the immune microenvironment in NSCLC and how to modulate this tumor immune microenvironment by RT to induce amplified antitumor immune responses to ICIs in advanced NSCLC patients.

In the first part of this thesis, a multiangular approach of a combination of protein and mRNA expression with clinicopathological characteristics in a large cohort of early stage, resected NSCLC samples will be discussed. The second part focusses on the immune modulating effects of RT, in particular when combined with immunotherapy treatment in metastatic NSCLC.

PART I. Exploring the tumor immune microenvironment

Expression of PD-L1 assessed by IHC is still the most important clinical biomarker to predict response on ICI in NSCLC, but specificity and sensitivity are relatively low. In **chapter 2**, we explored mechanisms of PD-L1 upregulation or to be more precise the lack thereof by comparing PD-L1 expression in tumor cells vs. immune infiltrating cells in early stage resected NSCLC samples. Not only T cells and the PD-1/PD-L1 pathway play a significant role in tumor-immune interactions. In **chapter 3**, an unsupervised exploration based on an expression of a wide variety of immune genes was performed in the same resection cohort, leading to the discovery a 34-gene signature with strong prognostic power in SCC, but not AD.

PART II. Modulating the tumor immune microenvironment

Although long-lasting clinical responses have been observed in responders, only a minority of NSCLC patients respond to ICIs monotherapy. **Chapter 4** provides a review of the immunoediting ability of RT, relevant pre-clinical and clinical data concerning the abscopal effect of the combination of ICIs with RT with a focus on NSCLC. In **chapter 5**, we present the results of the PEMBRO-RT trial, where advanced NSCLC patients were randomized between pembrolizumab alone vs. pembrolizumab after stereotactic

body radiation (SBRT) to a single tumor site. The PEMBRO-RT trial showed benefit of the combined strategy over pembrolizumab alone, but this did not meet our pre-emphasized criteria of meaningful clinical benefit. Finally, in **chapter 6**, a pooled analysis of the PEMBO-RT trial combined with a similar randomized trial performed at the MD Anderson Cancer Center is presented.

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