Immune Prophets of Lung Cancer: The Prognostic and Predictive Landscape of Cellular and Molecular Immune Markers1,2

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Abstract

Lung cancer is the leading cause of cancer deaths throughout the world. The majority of patients are diagnosed with locally advanced or metastatic disease when surgery, the best curative option, is no longer feasible. Thus, the prognosis of lung cancer remains poor and heterogeneous and new biomarkers are needed. As the immune system plays a pivotal role in cancer, the study of tumor microenvironment, with regard to the immune component, may provide valuable information for a better comprehension of the pathogenesis and progression of the disease. Through a detailed and critical evaluation of the most recent publications on this topic, we provide evidences of the prognostic and predictive significance of immune markers in tumor and in peripheral blood of lung cancer patients: from the landscape of immune cells (macrophages, neutrophils, lymphocytes and natural killer) and their cytokines, to the analysis of immune-checkpoints (PD-L1 and CTLA4), up to the genetic and epigenetic regulation of the immune response (immune gene signatures and miRNA). We also argue about the lights and shadows related to immune marker use in clinical practice, emphasizing on one hand the importance of their assessment in the choice of therapeutic treatment, on the other, the difficulty in their determination and reproducibility of literature data. The following review gives a foundation and a suggestion for future studies investigating tumor immunology in lung cancer.

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Introduction

Immunosurveillance in Lung Cancer: The Prognostic Role of Tertiary Lymphoid Structures (TLSs)

Almost 50 years passed since Burnet first introduced the concept of immunosurveillance [1], refined later in immuno-editing by Dunn and colleagues [2]. According to the immunosurveillance theory, the host can control tumor growth through the activation of adaptive and innate immune mechanisms, during the early stage of cancer (elimination phase). Under the constant immune pressure (continued deletion of cancer cells recognized by the immune system), some tumor cells undergo genetic and epigenetic changes (immune-editing), enabling them to avoid immune attack. Tumor escape occurs when neoplastic cells evade immunosurveillance and the tumor microenvironment (TME) provides a survival advantage for neoplastic cells. As for other cancers, the concept of the immune-editing can be applied to the lung cancer [3]; thus, the immunosurveillance of lung cancer can be effective in early oncogenesis but it is inhibited in cancer progression, developing a clinically detectable tumor. Evidence for immunosurveillance in lung cancer lies firstly in the proper histology of lung; secondly in the large body of scientific literature demonstrating an immune infiltrate of adaptive and innate immune cell populations [4]. The lung is a mucosal surface of the body, exposed constantly to inhaled particles including pathogens, as well as other potential toxins [5]. Lung protects itself using local tissue structures such as the mucus layer, ciliary ladder, and smooth muscles. Moreover, the respiratory epithelium is also able to directly sense pathogens and respond by releasing antimicrobial molecules able...
to opsonise bacteria. These innate processes are usually able to maintain sterility of the lung without the intervention of immune system cells. The latter are the next line of defense in the lung. Indeed, pulmonary immune homeostasis is maintained by a network of tissue-resident immune cells that continually monitor the external environment [5]. In health conditions, they contribute to tolerance to innocuous inhaled particles, while ensure an efficient and rapid immune response against invading pathogens. Immune cells of lung tissue are heterogeneous and involve alveolar macrophages, dendritic cells (DCs), and lymphocytes. CD8+ T cells and CD4+ T cells are the most prevalent subtypes of lymphocytes in lung tissue, although natural killer (NK) cells and NKT cells are also present. Very few B cells were found in the lung. The major part of CD4+ subset in the lung are T helper 1 (Th1), while T helper 2 (Th2) and regulatory T cells (Tregs) were detected at low levels [6]. Lung mononuclear phagocytes have been shown to adapt specifically to the lung environment, and contribute to lung homeostasis, scavenging, and immunosurveillance [7]. In lung cancers these immune cells are highly organized in ectopic lymph- node-like structures, called TLSs, not present under normal conditions [8]. TLSs resemble and function like secondary lymph-nodes, and antigen presentation take place in them. TLSs are considered a gateway for the entrance of immune cells from the blood to the tumor, through specialized blood vessels, named endothelial venules, which surrounded TLSs [8]. The role of TLSs in the immunosurveillance is supported by a positive correlation between high density TLSs, containing CD8+ T cells, and improved survival of patients, also suggesting a good prognostic value of infiltrating CD8+ T cells in lung cancer [9]. Interestingly, other authors found that patients with few TLSs, but high number of infiltrating CD8+ T cells, had poor survival, underlying the importance of TLS structures themselves; in these structures CD8+ T cells alone were not capable to satisfactorily fulfill their antitumor role without mature DCs [10]. Moreover measurable IgG and/or IgA versus tumor antigens, have been isolated from TLSB cells [8]. In all cases, in lung cancer, the density of TLSs correlates with a favorable prognosis.

**Prognostic Immune Cells in Lung Cancer**

Tumor infiltrating immune cells as macrophages, neutrophils, lymphocytes, have a pivotal contribution in cancer progression and critically influences the clinical outcome of patients depending on density and localization. In Figure 1 we can see a representation of the positive and negative prognostic significance of immune cells in lung cancer microenvironment.

**Tumor Associated Macrophages (TAMs)**

Given the prevalence of macrophages in the lung, our knowledge of TAMs and the spectrum of macrophage phenotypes (tumor suppressing, M1; tumor-promoting, M2) has progressed over the past decades [11,12]. This can be seen in the evolution of studies investigating macrophage infiltration as a prognostic indicator of lung cancer [13]. Some papers demonstrated that high numbers of macrophages in tumor islets were positively correlated with favorable clinical outcomes and longer survival, in both surgically resected and advanced-stage lung cancers, whereas high numbers of macrophages in the tumor stroma were negatively correlated with patient outcome [14,15]. However, the prognostic significance of tumor islet or stromal TAMs, lacked consensus of another study reporting no association with survival [16]. Of note, these studies differed in the methodology used (tissue microarrays or whole sections, score, antibodies used to mark macrophages as anti-CD163+ instead of anti-CD68+). To more accurately define macro-

**Tumor-Associated Neutrophils (TANs)**

TANs represent a significant portion of tumor-infiltrating cells and accumulate in many types of cancers including lung cancer [19]. It has been hypothesized that TANs polarize into either an N1 antitumoral or N2 protumoral phenotype, in response to cancer epithelial- and stromal-derived signals [20]. CD66b+ is an established marker of TANs, stored in neutrophil granules and constitutively expressed by human neutrophils [21]. The prognostic role of CD66b+ TANs has been associated with unfavorable outcome for a number of malignancies [22,23]. In non–small cell lung cancer (NSCLC), two previous studies failed to reveal significant association between TANs and patient outcome [24,25] but none of these evaluated cancer histological subtypes. Recently Rakae et al. conducted a study on 536 NSCLC patients of which 172 harbored lymph node metastases [26]. The authors demonstrated that high intratumoral CD66b+ TAN density in squamous cell carcinoma (SCC) subgroup, was an independent positive prognostic factor for disease-free survival; by contrast, in adenocarcinoma subgroup, high intratumoral TAN density was an independent negative prognostic factor [26]. Likewise, in patients with lymph node metastases, high level of intratumoral TANs was associated with poor prognosis. Differently, stromal CD66b+ TANs were not associated with outcome of NSCLC patients [26]. Eruslanov et al. demonstrated that in early stages of lung cancer, the cross talk between TANs and distant activated T cells led to the up-regulation of CD54, CD86, OX40L and -1BBL, costimulatory molecules on the neutrophil surface, which activated T cell proliferation in a positive-feedback loop [27,28]. Considering the results of these studies, we think that in the earliest stage of lung cancer TANs are not immunosuppressive, but stimulate T cell response, while in advanced lung cancer their phenotype changes supporting the tumor.

**Tumor Infiltrating DCs (TIDGs)**

DCs represent a heterogeneous and highly plastic immune cell system with a central role in controlling immune responses. In cancer, DCs are able to take up and process apoptotic and necrotic tumor fragments and present tumor antigens to antigen-specific helper and cytotoxic T cells. In this interaction, the mature DCs crucially need to display T-co-stimulatory molecules (CD40, CD86) that will favor cytotoxic T-cell responses. Accordingly, the intratumoral infiltration and activation status of DCs are emerging as clinically relevant parameters in lung cancer, having a substantial prognostic impact. Got et al. in 458 NSCLC lesions found that a high density of mature DC (DC-lamp+) in the TLSs correlated with infiltration of the lesions by T cells and expression of immune-related genes indicating T-cell activation, T helper 1 phenotype and cytotoxic differentiation [9]. A high density of TLS-associated DCs was also associated with improved survival [9]. However, the majority of TIDCs, in resected lung cancer specimen, was shown to reside in an immature state, to strongly overexpress the T-cell inhibitory molecule PD-L1 [29,30] and PD-L2 [30], and to acquire classical surface markers and functions commonly
ascribed to TAMs and myeloid-derived suppressor cells. These TIDCs are capable of actively suppress T-cell function through the secretion of Arginase-1 or indoleamine 2,3-dioxygenase. Thus, compare to peritumoral lung tissue, lung tumors are heavily infiltrated by cells sharing prototypical markers of CD11b+ DCs and M2-polarized/tumor-supporting macrophages, with high cell surface levels of PD-L129. Furthermore, low expression of IL-12 and of the co-stimulatory molecules CD80/CD86 in lymph nodes draining lung adenocarcinoma predict a poorer outcome [29]. Finally, Pyfferen et al. demonstrated that TIDCs-associated miRNA signatures have a negative prognostic impact in NSCLC [29].

**Tumor infiltrating lymphocytes (TILs)**

TILs are a heterogeneous population of tumor microenvironment comprising mainly T lymphocytes and to a lesser degree B lymphocytes and NK cells. According to the cell surface markers, T lymphocytes include CD8+ cytotoxic T lymphocytes (CTL), CD4+ T helper lymphocytes (Th), CD45RO+ memory T cells (Tm) and FOXP3 regulatory (Tregs) cells. The correlation between TILs and clinical outcome of patients has been extensively studied in lung cancer. Many studies acted to demonstrate the prognostic role of TILs in lung cancer have been published from 2003 to 2014 [31–35]. However, these studies reported contradictory results with limited statistical power, due to multiple factors examined, or to small number of patients, and they were non homogeneous with regard to stage, histological types of lung tumor and distribution site of lymphocytes (stromal lymphocytes, sTILs and intratumoral lymphocytes, iTILs). Fortunately in 2015 Geng et al. made clarity, publishing an excellent meta-analysis of studies investigating the prognostic impact of TILs in lung cancer patients. This study included 29 reports involving 8600 patients with NSCLC [36].
The statistical results confirmed that high density of TILs was associated with favorable progression free survival (PFS), rather than OS. Subgroup analysis was performed according to TIL subsets including CD8+, CD4+ and FOXP3+ T cells and reported a better OS in patients with high level of CD8+ T cell infiltration in tumor stroma (TS) and tumor nest (TN), and in both TS and TN. Compared with CD8+ T cells in TN, the prognostic effect of CD8+ T cells in TS appeared more significant. High density of CD4+ T cell infiltration in TS, rather than in TN, was associated with better prognosis in lung cancer. By contrast high density of FOXP3+ T cell infiltration in TS could be recognized as a negative prognostic factor for NSCLC [36]. More recently Zeng et al. published another meta-analysis study, on the prognostic value of TILs in NSCLC, included sixteen reports of the Geng’s study plus 8 additional studies [37]. The authors showed results overlapping those of Geng’s study underlying TILs have a prognostic significance for both OS and recurrence [37]. Among the meta-analysis studies reported by Zeng, very interesting, for what concern the quantification of TILs, was that of Schalper et al. who used multiplex quantitative immunofluorescence to measure the level of CD3+, CD8+ and CD20+ in 552 NSCLC patients [38]. The level of TILs was obtained in different tumor compartments by using cytokeratin stain to define tumor cells, and 4′,6-Diamidino-2-Phenylinodol. The authors found that increased levels of CD3+ and CD8+ TILs were associated with better outcome in NCLSC [38]. In the majority of published papers, T cell subsets were assessed by immunohistochemistry (IHC) with a manual semi-quantitative approach. Different cut-off were used to define “low” and “high” for each marker and for the epithelial/tumor nest compartment and the stromal compartment, according to the staining distribution [32]. Of note, the study of Donnem et al., who recently demonstrated that the stromal CD8+ T cell density, scored on a manual semi-quantitative 4-point scale, had an independent prognostic value and could stratify patients within each tumor/lymph node/metastasis (TNM) stage [39]. This paper was followed by a second study and proposal to introduce an IHC-based “TNM immune” staging system into clinical use for NSCLC [40]. After these two big meta-analysis studies, all researchers have oriented their studies in order to propose a reproducible method for TIL quantification in lung tumor microenvironment, that could clarify their prognostic and predictive role. One of these, is the study of Brambilla et al. [41]. For these authors the discrimination between stromal and epithelial infiltration may add more confusion than precision, due to the lack of inter-observer reproducibility, and from a pathology point of view adds little, because by definition, the tumor environment includes stroma [41]. Finally the last relevant publication concerning this topic is by Obeid and colleagues [42]. The authors assess 9 sampling strategies of 23 primary NSCLCs with the purpose to evaluate which of these 9 methods had the closest correlation with CD8+ TIL density measures of a whole tumor section, as well as with survival outcomes. The strategy showed the greater concordance with whole tumor, was that used multiple random samples of 20% of the tumor or a random core biopsy measuring 10x1mm [42]. The authors found that patients who had higher CD8+ counts in the center of the tumor experienced longer OS than those with low CD8+ counts [42].

Anticancer immune response is to date especially investigated for NSCLC, without regard to histological types. Recently it was reported a significant lower proportion of CD8+ cells and higher FOXP3+/CD8+ ratio in metastatic versus free lymph nodes only in adenocarcinoma, indicating a particular biology of this type of NSCLC [43]. Other authors demonstrated that CD8+ T cell infiltration strongly contributed to a better prognosis in adenocarcinoma when tumor cells retained the expression of classical HLA class I and did not express HLA-E [44]. Therefore, analysis of HLA-A, -B/C and HLA-E expression should be included as biomarkers together with CD8+ analyses, to predict the response to immunotherapy. In relation to small cell carcinoma (SACC), it has demonstrated the prognostic role of T memory CD45RO+ cells. These cells alone and in combination with CD8+ TILs, in tumor and stromal compartments and within each pathological stage (from stage I to IIIA), were a significant prognostic indicator of improved survival time [45]. The putative contribution of NK cells to immunosurveillance in lung cancer has been an ongoing topic. Some studies reported that NK infiltrating the tumor tissue were associated with better prognosis in several tumors, included lung cancer [46]. Other studies refer to the prognostic role of peripheral blood rather than tumor tissue NKcells; thus we reserve to discuss it in the next paragraph.

**Prognostic Immune Circulating Markers in Lung Cancer**

**Immune Circulating Cells**

For lung cancer <30% of the tumors are resectable and available for a complete microscopic examination. In other cases, the material for the study of inflammatory infiltration may be a tumor biopsy. Histological biopsies or cytological samples are too small and not representative for evaluating inflammatory infiltration. The immune response may be evaluated easier by peripheral blood examination, although it reflects systemic changes, different from local ones.

Neutrophils account for the most peripheral white blood cells [47]. A series of studies have explored the correlation between peripheral neutrophils and lymphocytes ratio (NLR) and the prognosis of lung cancer. A meta-analysis, including 14 studies and 2735 lung cancer cases, showed that high NLR yielded a worse OS in NSCLC and SCLC [48]. These data agree with those recently published by Deng et al [49]. The latter reported that elevated pretreatment values of NLR were an independent factor for poor prognosis in SCLC patients. Moreover high platelet and lymphocytes ratio were associated with poor prognosis too [49]. More recently Akinci Ozuyrek et al. demonstrated that NLR was more significant in determining the prognosis in NSCLC than in SCLC cases [50]. Wang et al. studied, by flow cytometry, T lymphocyte subgroups in peripheral blood of NSCLC patients and healthy adults, evaluating their clinical significance in diagnosis, treatment and prognosis [51]. CD3+, CD4+, CD8+ ratio and NK cells in NSCLC were decreased significantly in comparison with the control group, and their levels inversely correlated with the clinical stage of NSCLC, decreasing with the increase of clinical stage; CD8+ cells demonstrated no significant change and Treg cells were significantly more frequent than in the control group, and increased with the clinical stage of NSCLC [51].

NK activity was also related to lung cancer prognosis [46] and peripheral NK cell cytotoxicity was reduced in lung cancer patients [52]. Xu et al. demonstrated that the overexpression of T cell immunoglobulin and mucin-domain-containing molecule-3 (Tim3), on CD3+ CD56+ NK cells and CD3+ CD56dimNK subset, was associated with lymph node metastasis, and a shorter OS of patients with lung adenocarcinoma [53]. Other authors demonstrated an aberration of NK cell function in NSCLC. This aberration consisted in a constitutively low expression at the mRNA level of the three NK isoform receptors (NCR1/NKP46; NCR3/NKp30; NKp30), which correlated with poor OS and PFS [54].

For deeper and specific characterization of local immune response, the analysis of bronchoalveolar lavage (BAL) fluid may be used [55]. In
the BAL obtained from a lung afflicted by cancer, the following changes may be observed: increased number of neutrophils, predominance of T-cells and cytotoxic CD8+ T cells, prominent percentage of Tregs, polarization of macrophages to the M2 population, significantly increased concentration of TGF-β [56]. These alterations were significantly different than peripheral blood and BAL material obtained from the healthy lung, symmetrically to tumor localization in the lung afflicted by cancer.

**Interleukines and chemokines**

Cancer cells communicate with the microenvironment via a complex network of many growth factors, chemokines, interleukines and their own receptors. In the last decades several studies focused on the association between the expression level of interleukines, measured in serum, BAL and in lung tumor tissue, and patient survival or progression. Interleukin-20 (IL-20) and interleukin-22 (IL-22) have modulatory and opposing effects on cancer cells: IL-20 is an inhibitor of angiogenesis, while IL-22 stimulates tumor growth [57]. Recently, Naumnik et al. found that IL-20 and IL-22 in the serum and BAL of NSCLC patients are prognostic factors of cancer progression [58]. The authors reported high serum levels of IL-20 were negatively associated with cancer progression, while they failed to find an association between survival and serum levels of IL-22. Moreover the found that lower levels of IL-22 in the BAL of NSCLC patients, compared to healthy control, were associated to worse survival. A possible explanation of this founding could lie in the distribution of IL-22 receptors. IL-22 receptor 1 (IL-22R1) is expressed exclusively on epithelial and tissue cells of lung cancer. The BAL concentration of IL-22 in NSCLC patients could be reduced due to its binding to the receptor [58]. Increased levels of IL-17 have been found in advanced NSCLC. Lin et al. demonstrated that IL-17 levels were significantly elevated in the serum of SCLC patients and correlated with tumor metastasis, stage, and shorter OS [59]. Thus IL-17 may be a novel prognostic biomarker in SCLC [59]. Other authors emphasized the importance to identify a “combined cytokine prognostic classifier” to detect patients at high risk of recurrence of lung cancer, thus requiring more aggressive treatment regimens at the time of diagnosis [60,61]. These authors firstly found that the high combined expression of IL-8 with IL-6 [60], and secondly of IL-6 with IL-17 [61], was negative prognostic factors for stage I lung cancer. In addition to interleukins, also chemokines and/or their receptors expression have been correlated with patient survival or progression in lung cancer. Moreover, most of the studies evaluated chemokine expression in tumor or stromal compartment than in patient serum. A very recent and exhaustive review on the role of chemokines in NSCLC was recently published [62]. In summary, in these patients, high levels of C-C motif chemokine ligand 2 (CCL2), CCL19, CXCL16, and low levels of CCL5 were associated to a better survival; high levels of C-X-C motif chemokine ligand 8, CXCL8, and C-X-C motif chemokine receptor 4, CXCR4, were associated to a worse survival [62].

**Immunoocheckpoints as Prognostic and Predictive Immune Markers in Lung Cancer**

Immunoocheckpoint are pathways that induce costimulatory and inhibitory signals, crucial for regulating the physiologic T cell immune response, maintaining self-tolerance and inducing tumor escape from immunosurveillance [63]. **Programmed death protein 1 (PD1)** and **Cytotoxic T-lymphocyte-associated protein 4 (CTLA4)** pathways, are considered the main checkpoints for effective immunotherapy in solid tumor and also in lung cancer (Figure 2). **PD1/PD-L1 axis**

PD1 is expressed by activated T cells, B cells, NK T cells, and myeloid cells and is often highly expressed by TILs [62]. PD1 ligand (PD-L1) is expressed by cancer cells, and can be up-regulated on TAM, DCs, fibroblasts, and activated T cells [64]. The expression of PD-L1 on tumor cells was demonstrated on cell membrane, in the cytoplasm, or both, in focal or scattered pattern. Ligation of PD-L1 with PD1 mediates suppression of T cell function, differentiation and survival [64]. In NSCLC, it has been reported that 20–60% of tumors were positive for PD-L1 and/or for PD-L2, at lower frequency [3]. PD-L1 is not only membrane bound, but also secreted as a soluble form (sPD-L1). In lung cancer, the source of sPD-L1 could be the tumor cell, or the tumor-infiltrating immune cells [64]. Soluble PD-L1 may compete for PD1 ligation with anti-PD1 monoclonal antibodies. Although some studies reported high PD-L1 expression in lung tumor cells or TILs, are predictive of the response to PD1 pathway inhibitors, PD-L1 has not proved adequately reliable as a single biomarker [64]. One explanation may be the current use of non-standardized IHC techniques for measuring PD-L1 levels in tissue. Different commercial anti-PD-L1 antibodies (Dako 28–8, Dako 22C3, VentanaSP142, Ventana SP263) have been developed and validated in clinical trials. The American Association for Cancer Research (AACR) and the International Association for the Study of Lung Cancer (IASLC), together with four pharmaceutical companies (Brystol-Meyers, Merck, Genentech/Roche and AstraZeneca) and two diagnostic companies (Dako and Ventana), in the Bluprint Project, compared four PD-L1 assays on the same set of lung cancers. The results were similar for the 22C3, 28-8 and SP263 PD-L1 antibodies, while PD-L1 expression, tested by SP142 antibody, was generally low [65]. The difference may be explained by the binding of PD-L1 extracellular domain for the three antibodies and PD-L1 cytoplasmic domain for SP142. Moreover, when the results of the different assays were translated into “positive” or “negative”, based on the cut-offs, only the 50% of specimen showed the same results for all tests. Recently Brody et al. published a meta-analyses study to clarify the prognostic role of PD-L1 in advanced NSCLC. A total of 35 eligible studies were selected for analysis. Among these, three large meta-analysis studies concluded that high tumor PD-L1 expression was associated with shorter survival [66]. A possible link between PD-L1 expression and poor prognosis in advanced NSCLC fits with the role of PD1/PD-L1 in suppressing anti-tumor response. Paulsen et al. showed that a low density of PD-L1+ stromal immune cells and PD1+ intraepithelial TILs+ predicted for unfavorable survival outcome, especially for patients with SCC [67]. By contrast, Velcheti and colleagues in a multivariate analysis of 544 patients (Stage I-IV NSCLC) reported a significant association between PD-L1 expression with increased TILs, and longer OS [68]. Similar results were found by Cooper et al. who demonstrated PD-L1 expression in ≥50% of NSCLC (Stage I-III) tumor cells was associated with longer OS, with the exception of adenocarcinoma patients [69].

The value of PD-L1 as a predictive biomarker for the therapy with anti-PD1/PD-L1 agents (nivolumab, pembrolizumab/atezolizumab, durvalumab), was evaluated in 16 studies of Brody’s meta-analyses [66]. Among nivolumab monotherapy studies, 3 reported greater treatment benefit in patients with high versus low tumor PD-L1 expression. Conversely, a randomized controlled trial in squamous NSCLC patients concluded that the treatment benefit of nivolumab was independent of tumor PD-L1 expression. For pembrolizumab, high PD-L1 expression was correlated with improved treatment effect in 2 studies. Three of 4 atezolizumab studies, reported that higher
PD-L1 expression levels were associated with greater treatment effects and longer survival. For durvalumab monotherapy, a Phase 1/2 study demonstrated that the overall response rate was higher in patients with high versus low tumor PD-L1 expression [66]. The Food and Drug Administration (FDA) approved nivolumab, pembrolizumab and atezolizumab for the treatment of NSCLC patients with disease progression or after platinum-based chemotherapy. Furthermore, in October 2016 pembrolizumab was approved by the FDA as a 1st line treatment for advanced NSCLC patients, based on the results of the clinical trial KEYNOTE-024 that showed significantly improved response rate, PFS and OS when advanced NSCLC patients, whose tumors harbored PD-L1 expression by IHC in 50% or greater of tumor cells, were treated with this drug compared to platinum-based chemotherapy in the 1st line setting [70].

**CTLA4**

CTLA4 is an inhibitory molecule expressed on T cells involved in the negative regulation of T cell interaction with antigen-presenting cells (APC). It inhibits binding of CD28 on T cells, to B7 proteins on APCs, thus weakening the costimulation on T cells [71]. CTLA4 is also constitutively expressed on Treg and promotes their regulatory function [72]. CTLA4 was found on the cell surface and in the cytoplasm of tumor cells in about 50% of NSCLC patients. High CTLA4 expression, but not PD1, predicted worse survival in NSCLC and other malignancies, like nasopharyngeal or esophageal carcinoma [73]. Contrarily, other authors found CTLA4 overexpression correlated with good survival and reduced death rate in radically resected NSCLC [74]. Recently, Paulsen et al. evaluated by IHC CTLA4 expression in 536 patients with primary resected stage I-IIIA NSCLC [75]. CTLA4 expression in neither tumor epithelial cells, nor stromal cells, was significantly associated with disease specific survival. However, high stromal CTLA4 expression predicted improved disease specific survival in SCC subgroup. By contrast, CTLA4 expression in metastatic lymph nodes, was an independent negative prognostic factor [75]. Basing on these studies, we think that CTLA4 expression has diverging prognostic impact with regard to the histological lung tumor and stage of disease. The anti CTLA4 IgG1 humanized antibody, ipilimumab, binds to CTLA4 and prevents the inhibition of CD-28/B7 signaling. It leads to the reactivation of the antitumor immune response mediated by specific T cells and depletion of Tregs [76]. A phase 2 study with ipilimumab, in combination with chemotherapy, in advanced NSCLC patients, showed promising results with a significant improvement in PFS versus
the group treated with chemotherapy alone. However, actually there is no validated biomarker considered able to predict response to anti-CTLA4 therapy [76].

**Exosomes as Modulators of Lung Cancer Immune Response**

Exosomes are nanovesicles of 50-100 nm of diameter that are released from most viable cells and play an important role in intercellular communication. They are exocytosed in a constitutive manner in both physiologic and pathological conditions and can be found in several body fluids: urine, saliva, blood, BAL. Exosomes contain messenger RNA (mRNA), microRNA (miRNA), double-stranded DNA (dsDNA) and proteins that could serve as diagnostic, prognostic and predictive biomarkers for different tumors [77]. Many correlations were found between tissue and exosomal biomarkers in different cancers, included lung cancer [77]. Exosomes are reported to mediate lung cancer invasion, metastasis and drug resistance and to transport proteins (Epidermal growth factor receptor, EGFR, claudins, KRAS) and miRNA (tumor suppressor miRNA, non-coding RNA) related to poor OS of adenocarcinoma and NSCLC patients [77]. In lung cancer, tumor-derived exosomes disable anti-tumor immune effector cells and promote tumor escape from immune control. The EGFR carried by lung cancer cell exosomes, can induce immune-resistance of DCs and CD8+ T cells, by tumor-specific Treg cells, with immune escape of cancer cells [78]. Liu et al. demonstrated that tumor exosomal RNAs promoted lung pre-metastatic niche formation by the up-regulation of alveolar epithelial talk like receptor 3, TLR3, with neutrophils recruitment and increasing cytokines production [79]. By contrast, the exosome derived miR-302b suppressed lung cancer cell proliferation and migration through its interaction with the transforming growth factor β receptor II, TGFβRII, mRNA [80]. Beside tumor cell derived exosomes, these vesicles are also released by normal immune cells. Since 2003, it was demonstrated that lung cell-derived exosomes, present in healthy human BAL, express MHC class I/II and costimulatory molecules, suggesting their exocytosis from antigen presenting cells and activity as immunomodulatory agents [81]. DC-derived exosomes prime specific cytotoxic T lymphocytes and activate anti-tumor immune response [81]. The presence of MHC-I/MHC-II molecules, on the surface of dendritic cell-derived exosomes, facilitates the direct stimulation of CTLs and CD4 + T cells [82]. Therefore while exosomes deriving from lung cancer cells have a pro-tumor effect, exosomes from immune cells of lung tumor environment are useful tool for tumor antigen-specific immunity and may exhibit utility in lung cancer immunotherapy.

**Epigenetic Regulation of Lung Cancer Immune Response**

Advances in the field of lung cancer epigenetics, provide a very promising step towards the direction of novel biomarker development. Epigenetics consists of heritable modifications in the chromatin that influence gene expression without directly altering the DNA coding sequence [83]. Epigenetic mechanism can be grouped into DNA methylation, DNA acetylation (histone/nucleosome remodeling) and micro RNA (miRNA) [84].

**DNA Methylation in Lung Cancer and Immune Response**

Global hypomethylation is frequent in NSCLC and is associated with genome instability [84]. CpG islands methylation is completed by different DNA methyltransferase whose expression is implicated in the pathogenesis of lung cancer. A large number of aberrantly methylated genes have also been identified in lung cancer [84], among which, the immune-checkpoint genes. Marwitz et al. analyzed the epigenetic modification of PDC1 (PD1), CD274 (PD-L1) and CTLA4 in NSCLC tissue from 39 patients [85]. Results were correlated with transcriptome data. Significant differences in the CpG-methylation patterns between tumor tissues and controls were observed for CTLA4 and PD1: NSCLC tumors exhibited a decreased level of CpG methylation in these loci compared to tumor-free tissues, while no differences for PD-L1 could be observed [85]. Hypermethylation may also have significant in the pathogenesis of lung cancer. Recent data suggest that the modulation of DNA methylation via methyl transferase inhibitors might triggers anti-tumor immune responses. Wrangle et al. studied the expression signatures of immune genes and pathways activated in NSCLC by Azacytidine (AZA), an hypomethylating agent [86]. They found that the interferon regulating factor 7 (IRF7), an upstream activator of genes involved in type 1 interferon signaling, hypermethylated in lung cancer, was up-regulated by AZA [86]. These data suggest that IRF7 silencing by DNA methylation could result in suppression of immune-regulatory genes important for the immunosurveillance involved in cytotoxic immune mechanisms against cancer (Figure 3A).

**Histone Modification in Lung Cancer and Immune Response**

Histone epigenetic modifications play a crucial role in lung carcinogenesis. Histone deacetylases 1 and 3 (HDAC1, HDAC3) gene expression appears to correlate with lung cancer progression and poor prognosis in adenocarcinoma patients [83]. Histone deacetylase inhibitors (HDACIs), in addition to their direct anti-cancer effects, strengthen the immune system, by up-regulating the expression of MHC class I/II proteins, and co-stimulatory/adhesion molecules such as CD80, CD86, human leukocyte antigen (HLA-DR, HLA-ABC) and intracellular adhesion molecule 1 (ICAM-1) [83]. HDACIs may also enhance the immune response by altering the activities of immune cells, directly or indirectly through the modulation of cytokine secretion [87]. HDACI-treated NSCLC cells down-regulated tumor necrosis factor receptor 1 (TNFR-I) mRNA and surface protein expression, and responded to TNF-treatment with attenuated NF-kB nuclear translocation and DNA binding [88].

**MicroRNAs (miRNA) as Modulators of Lung Cancer Immune Response**

MicroRNAs are a class of small noncoding RNA of 18 to 25 nucleotides that post-transcriptionally regulate gene expression. miRNA play important roles in the regulation of immune responses in cancer cells [89]. Many different miRNA have been reported to be abnormally expressed or deregulated in lung carcinoma and to have diagnostic and prognostic value as biomarkers [90,91]. Some articles demonstrated a strictly association between PD-L1 expression and three different miRNAs: miR-200, miR-34 and miR-197 [92-94]. Chen et al. found that high PD-L1 expression in primary tumor cells was strongly associated with high epithelial-mesenchymal transition (EMT) score and could be determined by miR-200 expression [92]. These authors indicated that PD-L1 tumor expression in NSCLC was regulated by miR-200/148b-3p: miR-200 acted as a cell-autonomous suppressor of EMT and metastasis and directly targeted and inhibited PD-L1 [92]. Low expression of miR-200 was associated with increased PD-L1 expression and suppression of T CD8+ infiltration [92]. Thus the assessment of miR-200 expression may be useful for the therapy with anti-PD-L1 antibodies. Recently Cortez et al. found that miR-34 regulated PD-L1 expression binding the 3′UTR of PD-L1 mRNA [93]. They demonstrated that mutated TP53 NSCLC cell lines...
had significantly higher expression of PD-L1 and lower expression of miR-34a, but no differences in the expression of miR34b and miR34ccompared to cancer cells wild type for TP53 [93]. Fujita et al. showed a negative correlation between miR-197 and PD-L1 expression: lower miR-197 expression was associated with shorter OS of NSCLC patients [94]. It was suggested that miR-197 is involved in chemo-resistance, tumorigenesis and metastasis. Indeed, miR-197 knockdown led to cisplatin and paclitaxel resistance in four lung cancer cell lines, while miR-197 overexpression induced cell sensitivity to these compounds [94]. Hence, miR-197 may be considered an important predictive immune marker in lung cancer. Interestingly, PD-L1 mRNA has no complementary sequence to miR-197 region. This miRNA regulates the CD28 protein kinase regulatory subunit 1B. The latter phosphorylated STAT3 which bound PD-L1 promoter inducing its transcription. High levels of miR-9 in lung cancer cells correlated with the down regulation of MHC I, favoring tumor escape. Low levels of miR-141 in mice model of malignant pleural effusion, resulted in increased production of CXCL1 and Treg recruitment and migration. Abbreviations: PD1 = programmed death protein 1; CTLA4 = Cytotoxic T-lymphocyte-associate protein 4; IRF7 = interferon regulating factor 7; PD-L1 = programmed death protein ligand 1; CKS1B = CD28 protein kinase regulatory subunit 1 B; STAT3 = signal transducer and activator of transcription; CXCL1 = C-X-C motif chemokine ligand 1; CXCR2 = C-X-C motif chemokine receptor ligand 2.
Recruitment of effector lymphocytes (termed M/D/N, comprised genes involved in the activation and professional antigen presentation. Two gene signatures, predominated by genes belonged to the MHC class II family (HLA-B, HLA-C, HLA-G, HLA-J) and immunoglobulin encoding genes such as IGKC, IGHD, IGLC1, IGLJ3, IGHA1, IGHM, IGJ and IGK. One signature, termed B/P was composed of immunoglobulin encoding genes such as IGKC, IGHD, IGLC1, IGLJ3, IGHA1, IGHM, IGJ and IGK. One signature, termed B/M/D was predominated by genes belonged to the MHC class II family (HLA-DR4, HLA-DRB1, HLA-DPA1, HLA-DPB1, HLADQB1, CD74) consistent with roles in professional antigen presentation. Two gene signatures, termed M/D/N, comprised genes involved in the activation and recruitment of effector lymphocytes (CD84, CD86, CCR1), regulation of immune responses (IL1RB2, IL1RB4, CD300A), macrophage differentiation and function (CSF1R, CCL2, CD14, CD163, CYBB, CLEC4A, CLEC7A) and myeloid IgG receptor signaling (FCGRI1, FCGRI1B, FCGRI2A, FCGRI2B, FCGRI3A, FCGRI3B). Finally, one gene signature, termed D (Lipopolysaccharides, LPS), showed greatest enrichment in LPS-stimulated DCs and was composed of MHC class I family genes (HLA-B, HLA-C, HLA-G, HLA-J) and a large number of genes with direct roles in interferon signaling (IRF7, IRF9, STAT1, ISG15, OAS1, OAS2, OAS3, IFI35, IFI14, IFI6, IFI11, IFIT3, IFIT5, HERC5, HERC6, DDX58, DDX60). These markers of immune involvement were significantly associated with patient prognosis.

Conclusions

The microenvironment of lung cancer has a strong prognostic value. The analysis of the immune contexture of this tumor revealed a set of cellular and molecular immune markers which could effectively and reproducibly classify patients according to their survival. Immune markers may be combined with the standard pathological TNM classification to form a TNM-Immunoscore for lung cancer. Further studies investigating larger cohorts of patients, uniform in histology, stage, methodologies, and assessing tumor-immune system interactions are warranted to fully assess the prognostic/predictive power of these markers.

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