

Immunotherapy in non-small-cell lung cancer: a bridge between research and clinical practice

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Lung cancer has been historically considered a poorly immunogenic disease because of the few evidence of immune responses in affected patients and the limited efficacy of immunomodulating strategies. Recent understanding of the molecular mechanisms leading to cancer immune evasion has allowed the development of a new class of drugs called immune checkpoint inhibitors, which reactivate host responses with outstanding clinical benefits in a portion of patients with non-small-cell lung cancer. In this review, we briefly summarize the basis of immunogenicity and immune escape of cancer, with specific focus on non-small-cell lung cancer, mechanisms underlying immune checkpoint inhibitors efficacy and the most updated results on potential biomarkers, with the final aim of defining current unmet needs of immunotherapy in clinical practice.

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Lung cancer has been historically considered a poorly immunogenic disease because of the few evidence of immune responses in affected patients and the limited efficacy of immunomodulating strategies, including vaccines [1]. Nevertheless, the recent understanding of the molecular mechanisms leading to cancer immune evasion has allowed to develop a new class of drugs, known as immune checkpoint inhibitors (ICIs), able to reactivate host responses and to mediate outstanding clinical benefit in a sizable proportion of patients with non-small-cell lung cancer (NSCLC).

Until a few years ago, the only therapeutic option for patients with metastatic NSCLC was platinum-based chemotherapy or target therapy for adenocarcinoma subtypes harboring known molecular alterations in driver oncogenes (EGF receptor [EGFR]/anaplastic lymphoma kinase [ALK]) [2]. However, the significant success of ICI in melanoma has paved the way to the clinical testing of this strategy in other solid tumors, including NSCLC [3]. Surprisingly, the stimulation of antitumor immune response through PD-1/PD-L1 blockade resulted in a significant and durable disease control in a subgroup of patients with advanced NSCLC, with a remarkable impact on overall survival (OS) and notable improvement of patient quality of life.

On the basis of these initial results, PD-1/PD-L1 ICIs are currently recommended as new standard treatment for NSCLC patients. Particularly to date, we have three monoclonal antibodies (MoAbs), nivolumab, pembrolizumab and atezolizumab, approved by regulatory authorities in second-line setting, of which one, pembrolizumab is approved also as first-line therapy [4]. The most recent survival update of the CheckMate 003 Phase I study of nivolumab in pretreated NSCLC patients revealed that about 16% of patients are still alive after 5 years of therapy, suggesting that a small but significant subgroup of patients derive an impressive and durable benefit from ICIs [5]. Conversely, evidence emerging from randomized trials showed that about 50% of patients do not benefit from treatment [6–9].

Thus, the identification of predictive biomarkers is an urgent need, representing one of the main objectives of lung cancer translational research that could help oncologists to select which patients may really benefit from immunotherapy. Unlike target therapies, immunotherapy modulates a complex network of molecular and cellular pathways, making the discovery of predictive biomarkers a very hard process and a challenging task for translational research. In addition, the interaction between tumor and immune system observed in preclinical models may not reflect what occurs in cancer patients, thus it does not necessarily provide reliable information for human use. Hence, the process has to rely mostly on clinical setting and on the availability of biological samples collected in a systematic and standardized manner from defined patients subsets. This means that the scientific community should promote a new era of clinical trials aimed at studying biological samples for the identification of immune-related biomarkers that has to be timely addressed.

With the present review, we would like to briefly summarize the basis of immunogenicity and immune escape in human cancers, with specific focus on NSCLC, the mechanisms underlying ICI clinical efficacy and the most updated results in term of potential biomarkers, with the final aim of defining the current unmet needs of immunotherapy in clinical practice. The broad use of ICI that is presently applied to NSCLC offers oncologists the unique opportunity of actively contributing to these studies, for a bridge between research and clinical practice.

Basis of cancer immunogenicity & immune escape

The immune mechanisms leading to the induction of a response against the tumor cells are very complex, but they can be envisaged as the result of opposite forces regulating tumor/host interaction during the different disease phases. From one side, the immunogenicity of cancer cells, in other words, the expression of 'altered' proteins detected by the immune system as 'antigens', induces the priming of antigen-specific T-cells through the processing and presentation by dendritic cells (DC) of antigenic determinants in the context of the human leukocyte antigen (HLA) molecules. Antigenic determinants in NSCLC are provided by tumor proteins ectopically induced by neoplastic transformation (as for cancer/testis antigens) [10], epithelial molecules increased in expression density (EGFR, carcinoembryonic antigen (CEA), epithelial cell adhesion molecule (EpCAM), mucin1 (MUC1)) [11] or mutated proteins deriving from cancer genetic instability that leads to the generation of new amino acid sequences able to be recognized by T-cells as 'neoantigens' [12]. The latter groups, globally quantified by measuring the tumor mutational burden, are considered to provide the most immunogenic epitopes and to associate with the presence of more efficient antitumor immune responses in course of immunotherapy [13].

Upon the engagement of their T-cell receptor (TCR) with the antigen/HLA complex expressed on tumor cells, lymphocytes mediate tumor cell lysis through the release of cytolytic granules (containing perforin, granzyme B and proapoptotic molecules such as FasL and TRAIL) and stimulatory cytokines such as IFN- γ , IL-2 and TNF- α . Sign of this reactivity is the presence of a T-cell infiltrate, most commonly composed by CD8⁺ T-cells that is often detected in solid cancers, including NSCLC, as linked to good prognosis [14], indicating that even in patients with clinically evident cancer, an active immune response can contribute in slowing-down disease progression.

Nevertheless, when this immunity is unable to clear the tumor, it drives instead a sort of chronic 'evolutionary' process that causes the selection of immune-resistant tumor cell clones, according to a mechanism known as immunoediting, and including the onset of antigen- or HLA-loss tumor cell variants [15]. Concomitantly, multiple immune escape strategies take place within the tumor microenvironment (TME), mainly relying on two pathways: the upregulation on T-cells of immune checkpoints (PD-1, CTLA-4, TIM3 and LAG3) that, binding to cognate ligands also induced on tumor and stroma cells by IFN- γ , deliver inhibitory signals to T-cells causing their reversible paralysis (exhaustion) [16]; the accrual at tumor site of immune regulatory cells that exert suppressive activity on T lymphocytes and block their function in the attempt to maintain tissue homeostasis. To the latter belong regulatory T-cells (Tregs) and the large and heterogeneous family of myeloid-derived suppressor cells (MDSCs). Both cell subsets belong to the physiological process of immune homeostasis and represent the response of the host to chronic inflammation, aimed at contrasting persistent immune stimulation. Specifically, Treg (CD3⁺CD4⁺, FOXP3⁺, CD25 high) are cells devoted to control autoimmunity [17], and they result increased in number and activation state in blood, lymph nodes and tumor site of cancer patients, including NSCLC [18]. They exert immunosuppression on effector CD8⁺ T-cells through multiple pathways including blocking of IL-2 synthesis, release of T-cell inhibitory cytokines such as IL-10 and TGF- β , and high expression of CTLA-4. Tregs restrain T-cell activation and their depletion is associated with more effective and durable response to immunotherapy, particularly to CTLA-4 blockade [19].

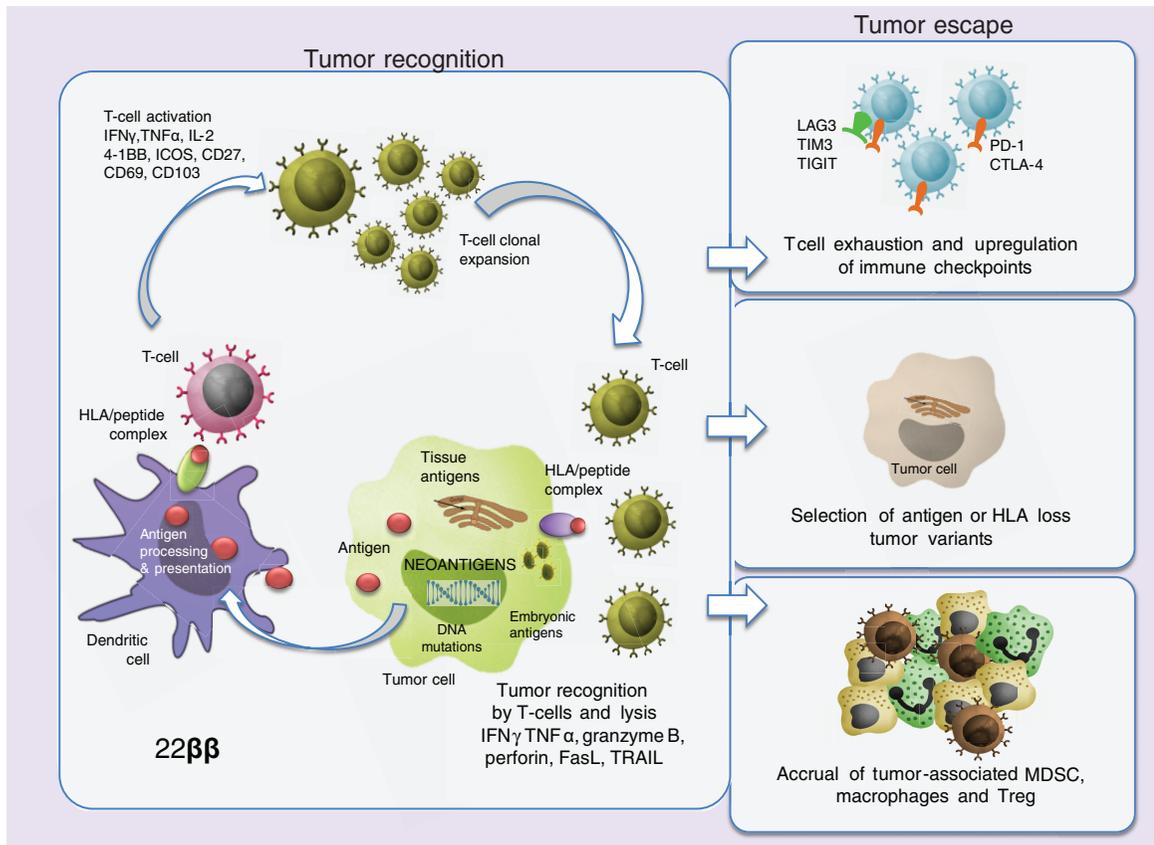


Figure 1. Basis of cancer immunogenicity and immune escape.

NSCLC lesions are also enriched in myeloid cells with respect to nontumor surrounding lung tissue, including cells of monocytic, granulocytic and macrophagic phenotype [18,20]. Aside from residential alveolar macrophages, most of these elements derive from the differentiation of immature myeloid cells coming from the blood and globally defined as MDSC [21]. It is indeed largely acknowledged that chronic immune stimulation, together with different cytokines and chemokines secreted by tumor cells, molds bone marrow myelopoiesis and promotes the mobilization of late precursors in the attempt to restrain inflammation and maintain tissue homeostasis. Pathological accrual of MDSC in blood, lymph nodes and TME of cancer patients, reproducibly occurring in association with poor prognosis [22] and limited response to immunotherapy, causes potent suppression of antitumor T-cells (via TGF- β , reactive oxygen species, nitric oxide synthase and indoleamine 2,3-dioxygenase [IDO]), stroma remodeling, VEGF-mediated angiogenesis and metastatic spread. These cells, defined as monocytic (CD14⁺HLA-DR^{neg}) or granulocytic (CD15⁺HLA-DR^{neg}) MDSC by cytofluorimetry, are measured as monocytes and granulocytes in routine blood test and this explains the negative prognostic impact that neutrophil or monocyte absolute counts, together with the relative ratio with lymphocytes, play in patients with cancer, including NSCLC [23,24] (Figure 1).

This complex immune scenario featuring TME has been hard to modulate in a consistent and pleiotropic fashion until few years ago, when immune checkpoints and their role in favoring tumor immune escape were discovered. CTLA-4 and PD-1, together with the cognate ligand PD-L1, are indeed the common denominators of many of the multiple immune cell subsets present in TME whose modulation, through antagonist MoAbs, has been recently shown to tilt the immune balance toward antitumor immune responses that can control disease progression in a substantial percentage of patients.

Mechanisms of action of PD-1 & PD-L1 inhibitors

CTLA-4 and PD-1 are two inhibitory receptors expressed on T-cells that, binding to their ligands (CD80/CD86 and PD-L1/PD-L2, respectively) expressed on several types of immune and tumor cells [25,26], play a central role in suppression of T-cell activity, thus regulating immune-response against tumor cells. CTLA-4 acts in the

activation stage of T-cell priming, which occurs in regional secondary lymphoid organs and is necessary to generate a population of effector T-cells against the tumor. On the other hand, PD-1 acts in the secondary stage of T-cell activation, binding its ligands on tumor cells and other stromal cells of TME [27,28]. In the last 10 years, many clinical trials on antibodies against CTLA-4, PD-1 and PD-L1 have shown encouraging antitumor activity in various malignancies, including NSCLC [22,29–33]. However, despite this major progress, many questions remain regarding the optimal use of ICI, as only approximately 30% of patients seem to benefit from this approach [34].

The blockade of PD-1 receptor can restore the activity of chronically exhausted tumor-specific T-cells. After binding with its natural ligands – PD-L1 (also known as B7-H1 or CD274) and PD-L2 (also known as B7-DC or CD273) – PD-1 receptor can negatively regulate the immune system, promoting self-tolerance and allowing tumor cells to escape from immune control [35]. PD-1 is a 288-aminoacid type-1 transmembrane protein of the immunoglobulin superfamily, encoded by the five exon *Pdcd1* gene located on chromosome 1 in mice and chromosome 2 in humans and normally expressed on B cells, T-cells and monocytes [36–38]. After interaction with its ligands, the intracellular domain of PD-1 is phosphorylated, leading to the downmodulation of the TCR signaling [39,40] and to a limitation of T-cell activity in the TME [41]. Moreover, also CD80 or B7-1 complex has been shown to bind PD-1, inducing inhibitory signals in T-cells with a subsequent anergic state [42,43]. The centrality of PD-1 in the immune regulation after chronic tumor antigen stimulation was confirmed by the first demonstration of PD-1 high expression on lymphocytes infiltrating human tumors activated by melanoma specific antigens, as reported by Fourcade *et al.* in 2009, and by its high presence on Treg cells that are among the most important CD4⁺ T-cells with suppressive function [44–50]. PD-L1 is the principal ligand of PD-1 receptor and is expressed on activated hematopoietic and epithelial cells, while PD-L2 is more present on DC and macrophages [26]. Immunohistochemical (IHC) assays have revealed high PD-L1 upregulation on cells of many different tumors including NSCLC [35]. Expression of PD-L1 on tumor cells is dependent on two mechanisms: an intrinsic expression correlated to various aberrant oncogenic signal pathways, such as phosphoinositide 3-kinase/protein kinase B, extracellular-signal-regulated kinases/mitogen-activated protein kinase and Janus kinase (JAK2)/STAT, and an induced expression dependent on inflammatory signals such as IFN- γ release induced by active antitumor immune response [32,51–54]. The adaptive mechanism seems to be more frequently involved in PD-L1 expression in TME, suggesting that the presence of PD-L1 is directly correlated to a previous activation of T-cells specific for a tumor antigen [55]. High PD-L1 expression was demonstrated on melanoma, ovarian, lung and other types of cancer cells [56–63]. Moreover, high PD-L1 expression was associated with strong presence of tumor-infiltrating lymphocytes (TILs) and IFN- γ in melanoma TME, explaining how activation of PD-L1/PD-1 pathway is an immune-resistance mechanism used by cancer cells to escape from immune-related destruction [53]. This can explain the rationale to use PD-1/PD-L1 ICIs in the treatment of tumors expressing high levels of PD-L1, with the aim to block tumor-induced immune suppression. In fact, MoAbs directed against PD-1 or PD-L1 can block T-cell inhibition mediated by PD-1 pathway, restoring immune response; many preclinical studies demonstrated the antitumor activity of these antibodies in murine tumor models, leading to further investigation in cancer patients [50,64]. Moreover, in preclinical setting, it has been demonstrated that also the Fc-gamma receptors of anti-CTLA-4 play a role *in vivo* models, increasing CD8⁺ to Treg cell ratio. These data suggest that, in highly immunogenic tumors, also the Fc-gamma receptors polymorphism status could be considered in the selection of patients likely to respond to anti-CTLA-4 [19].

ICIs in advanced NSCLC

On the basis of encouraging activity observed in early clinical trials, several MoAbs targeting PD-1 and PD-L1 have been investigated in clinical setting, showing a great clinical benefit in several tumor types, including NSCLC (Table 1).

Nivolumab (IgG4 anti-PD-1 MoAb) is the first ICI evaluated for safety and clinical activity in a Phase I dose-escalation expansion cohort study in patients with advanced heavily pretreated NSCLC. For all 129 patients, treated with different doses, median OS was 9.9 months (14.9 months in patients receiving nivolumab 3 mg/kg) and overall response rate (ORR) was 17%; squamous and nonsquamous histologies had similar efficacy [66]. This benefit was confirmed in two Phase III randomized trials (CheckMate 017 trial for squamous and CheckMate 057 trial for nonsquamous NSCLC). According to these findings, nivolumab was the first human anti-PD-1 MoAb approved by the US FDA for treatment of patients with metastatic both squamous and nonsquamous NSCLC who progressed after first-line therapy.

Pembrolizumab is a humanized monoclonal IgG4- κ isotype antibody against PD-1. In a Phase I expansion cohort trial (Keynote 001 trial) of pretreated and not-pretreated patients with advanced NSCLC, pembrolizumab,

Table 1. Anti-PD-1/PD-L1 immune checkpoint inhibitors approved for pretreated patients with advanced non-small-cell lung cancer.

Agent	Description	NSCLC setting approval	Trial	N. patients randomized	Outcomes	TrAE (G3-4) n. (%)	n (%) of TrAEs
Nivolumab	Fully human anti-PD-1 IgG4 monoclonal antibody	Stage IIIB or IV squamous-cell NSCLC after treatment with platinum-based chemotherapy	CheckMate 017 [7] Phase III Nivolumab 3 mg/kg q2w vs docetaxel 75 mg/mq q3w	272	OS: 9.2 vs 6.0 m; HR: 0.59; p < 0.001 RR: 20 vs 9%; p= 0.008 PFS: 3.5 vs 2.8 m; HR: 0.62; p < 0.001	All Fatigue Nausea Anorexia Pneumonitis Hypothyroid Diarrhea	58 (7) 16 (1) 9 (0) 11 (1) 5 (0) 1 (0) 8 (0)
		Stage IIIB or IV nonsquamous lung cancer after treatment with platinum-based chemotherapy and a tyrosine kinase inhibitor for EGFR/ALK-mutated patients	CheckMate 057 [6] Phase III Nivolumab 3 mg/kg q2w vs docetaxel 75 mg/mq q3w	582	OS: 12.2 vs 9.4 m; HR: 0.73; p = 0.002 RR: 19.2 vs 12.4%; p = 0.02 PFS: 2.3 vs 4.2 m; HR: 0.92; p = 0.39	All Fatigue Nausea Anorexia Pneumonitis Hypothyroid Diarrhea	69 (10) 16 (1) 12 (1) 10 (0) 1 (1) 7 (0) 8 (1)
Pembrolizumab	Humanized anti-PD-1 IgG4-j isotype monoclonal antibody	Advanced NSCLC after platinum containing chemotherapy or a tyrosine kinase inhibitor for EGFR/ALK-mutated patients, with at least 1% of tumors expressing PD-L1	Keynote 010 [8] Phase II/III Pembrolizumab 2 mg/kg vs pembrolizumab 10 mg/kg vs docetaxel 75 mg/mq q3w	1034	Pemb 2 mg/kg OS: 10.4 vs 8.5 m; HR: 0.71; p = 0.0008 PFS: 3.9 vs 4m; HR: 0.88; p= 0.07 Pemb 10 mg/kg OS: 12.7 vs 8.5 m; HR: 0.61, p < 0.0001 PFS: 4.0 vs 4.0 m; HR: 0.79; p = 0.004 RR: 18% vs 18% vs 9% (p < 0.001)	All Fatigue Nausea Anorexia Pneumonitis Hypothyroid Diarrhea All Fatigue Nausea Anorexia Pneumonitis Hypothyroid Diarrhea	63 (13) 14 (1) 11 (1) 14 (1) 5 (2) 8 (0) 7 (1) 66 (16) 14 (2) 9 (1) 10 (1) 4 (2) 8 (0) 6 (0)
Atezolizumab	Human anti-PD-L1 monoclonal IgG1 antibody	Stage IIIB or IV after one or two previous cytotoxic chemotherapy regimens (one or more platinum-based combination therapies)	POPLAR trial [65] Phase II Atezolizumab 1200 mg vs docetaxel 75 mg/mq q3w	287	OS: 12.6 vs 9.7 m; HR: 0.73; p: 0.04	All Fatigue Nausea Anorexia Pneumonitis Hypothyroid Diarrhea	67 (40) 20 (14) 12 (8) 17 (12) – – 7 (5)
			OAK trial [9] Phase III Atezolizumab 1200 mg vs docetaxel 75 mg/mq q3w	850	OS: 13.8 vs 9.6 m; HR: 0.73; p = 0.0003 PFS: 2.8 vs 4.0 m; HR: 0.95; RR: 14 vs 13%	All Fatigue Nausea Anorexia Pneumonitis Hypothyroid Diarrhea	64 (37) 26 (2) 17 (0) 23 (0) – – 15 (0)

ALK: Anaplastic lymphoma kinase; Doc: Docetaxel; EGFR: EGF receptor; HR: Hazard ratio; m: Month; NSCLC: Non-small-cell lung cancer; OS: Overall survival; Pemb: Pembrolizumab; PFS: Progression-free survival; q2w: Every 2 week; q3w: Every 3 week; RR: response rate; TrAE: treatment related adverse events.

at a dose of 2 or 10 mg/kg of body weight (mg/kg) every 3 weeks or 10 mg/kg every 2 weeks, showed a 19.4% ORR and 12.0 months OS in the overall population. In a subgroup of patients (n = 61) with a PD-L1 tumor proportion score of 50% or higher, the benefit was greater with an ORR of 41% [67]. According to this evidence and following Phase III randomized trial results, pembrolizumab has been recently approved as first-line treatment in patients with advanced NSCLC whose tumors have high PD-L1 expression (PD-L1 expression in >50% of tumor cells, as assessed by the assay IHC 22C3 pharmDx) [68] and in patients who have experienced disease progression after platinum chemotherapy, with at least 1% of PD-L1-positive tumor cells [8].

Atezolizumab is a humanized IgG1 MoAb-anti-PD-L1. Several clinical trials have demonstrated the efficacy of this agent in the treatment of NSCLC. In the first Phase I trial with an anti PD-L1 antibody, atezolizumab showed durable response in NCSCL patients [32]. In the Phase II randomized trial (POPLAR) patients with NSCLC who progressed on platinum chemotherapy and were treated with atezolizumab had a significant improvement in OS with a median of 12.6 months compared with 9.7 months of patients treated with docetaxel (hazard ratio [HR]: 0.73) with more benefit in patients with higher PD-L1 tumor cells expression and tumor-infiltrating inflammatory cells expression [65]. The following Phase III trial (OAK) [9] confirmed these results showing a significant reduction

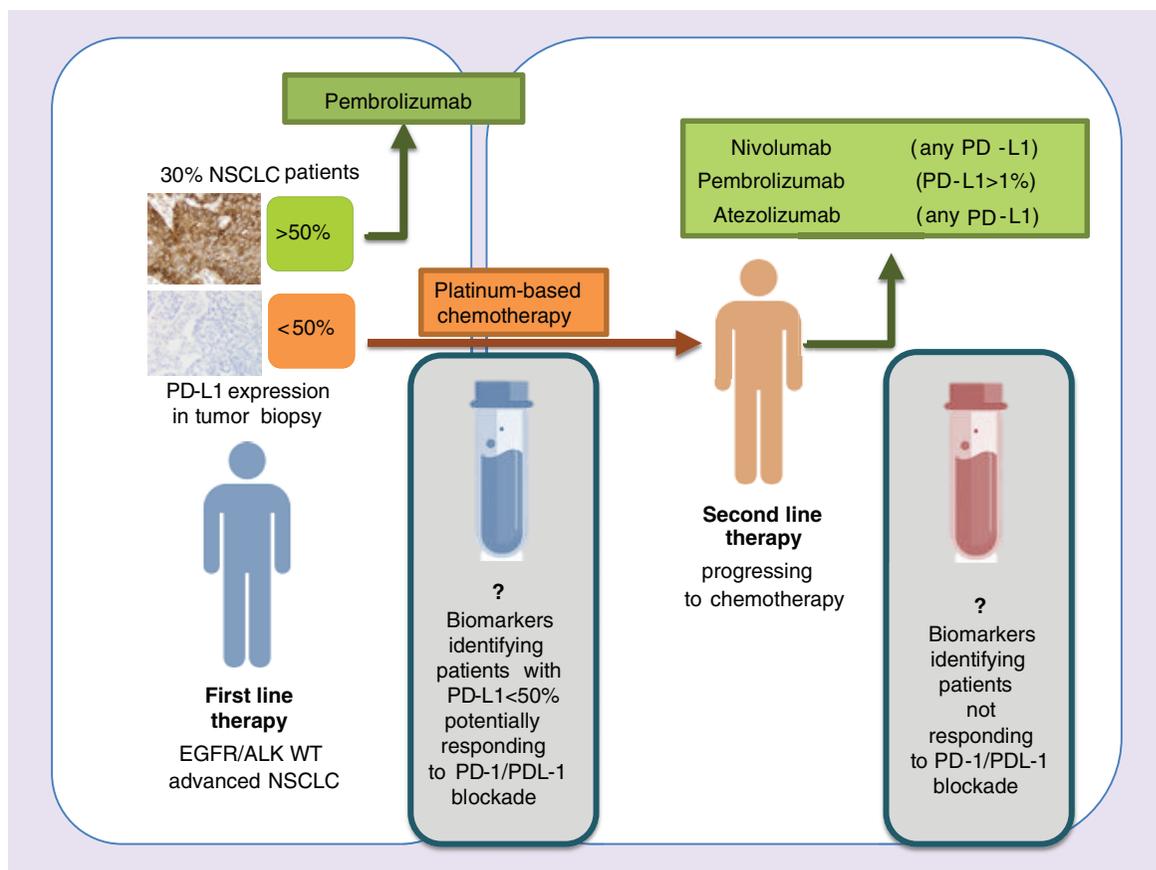


Figure 2. The clinical need of biomarkers to PD-1/PD-L1 blockade.

of death risk in the overall population, regardless of PD-L1 status, histology, age, smoking status and presence of CNS metastases, leading to the recent FDA indication for advanced NSCLC patients in second- or third-line setting.

Despite the significant survival benefit of ICIs for a large proportion of patients with advanced NSCLC, ORR is approximately 20% with a large proportion of patients not responding to these therapies [6,7,69]. Furthermore, a small proportion of them developed ‘hyperprogression’ to ICIs defined as a ‘RECIST’ progression at the first evaluation and as a ≥ 2 -fold increase of the tumor growth rate prior and upon anti-PD-1/PD-L1 therapies’ while ‘early deaths’ within the first 3 months of treatment with ICIs have been reported in about 10% of nonsquamous NSCLC patients included in the CheckMate 057 trial [70–72]. New clinical data are confirming the existence of this aggressive disease in a fraction of patients [70], making the identification of predictive biomarkers an urgent challenge for translational lung cancer research (Figure 2).

The role of PD-L1

Even if still limited by a lack of standardization in testing methods, the IHC assessment of tumor PD-L1 expression remains the only predictive biomarker approved for clinical setting [4]. From clinical datasets, it is becoming clearer that tumor PD-L1 expression facilitates the selection of subjects who are most likely to respond to ICIs. The Keynote 024 study clearly demonstrated that the presence of tumor PD-L1 expression more than 50% predicts pembrolizumab efficacy in first-line setting [68]. Conversely, a lower cutoff point of 5% used in the CheckMate 026 study of nivolumab versus platinum chemotherapy failed to demonstrate any predictive role, suggesting that expanding the population assigned to ICIs may increase the risk of including patients who are less likely to benefit from PD-1 inhibition. In light of these data, the anti-PD-1 pembrolizumab is the only ICI currently approved as upfront therapy in about 30% of patients with newly diagnosed, advanced NSCLC with PD-L1 expression more than 50%. Thus, the detection of PD-L1 expression on tumor cells should be always determined in order to decide

the best upfront treatment, at least until a prospectively evaluated alternative biomarker will show similar predictive value. Differently from first-line, PD-L1 status alone is not an appropriate biomarker to exclude pretreated patients from ICIs. Indeed, the majority of trials in second-line setting showed that, even if the survival advantage obtained with anti-PD-1/PD-L1 inhibitors increased according to a higher tumor PD-L1-expression, a significant number of PD-L1-positive patients did not respond to treatment, while about 30% of PD-L1-negative patients benefited more from ICIs as compared with standard chemotherapy with docetaxel [6–9]. These data clearly suggest that the correlation between tumor PD-L1 expression and the efficacy of these agents is imperfect, varying among different drugs and tumor subtypes, and that this could be attributed to different factors. A major issue emerging from these studies is represented by the heterogeneity of the IHC cutoffs, ranging from more than 1 to 50%, as well as the multitude of detection methods based on different PD-L1 IHC MoAbs used to define positivity in tumor specimens. In this regard, the International Association for the Study of Lung Cancer Pathology Committee is currently working to standardize and validate a unique and reproducible IHC test for PD-L1 assessment in clinical practice [73]. The preliminary results of the International Association for the Study of Lung Cancer Blueprint Project, in line with other European projects, showed that three of four analyzed PD-L1 detection platforms had similar analytical performance, reaching an overall percentage agreement of about 90%, even with some cases of discrepant results [73–75]. In addition to such technical issues, some biological aspects need to be also pointed out. Recent studies revealed that PD-L1 expression on the tumor cell surface may be regulated by two main mechanisms: the ‘inflammation-driven’ PD-L1 expression, consisting of an IFN- γ -mediated adaptive upregulation of PD-L1, associated with a high density of intratumor IFN-producing CD8⁺ T-cells, and an inflamed TME, revealing the presence of a preexisting suppressed immune response which may be reactivated by ICIs. Conversely, the ‘oncogene-driven’ PD-L1 expression is constitutive, not related to inflammation and associated with neither an immune response nor a T-cell tumor infiltration, thus less likely to respond to anti-PD-1/PD-L1 therapies [76,77]. A study by Ngiow *et al.* showed that tumors resistant to immunotherapy were infiltrated by PD-L1⁺ exhausted T-cells having also an overexpression of different inhibitory receptors other than PD-L1, such as TIM3, CTLA-4, LAG3 and B and T lymphocyte attenuator, and that the number of inhibitory receptors in the same CD8⁺ T-cells can affect the severity of T-cells dysfunction [78]. Finally, it is well known that PD-L1 is a dynamic biomarker, subjected to both space (primary vs metastatic lesions) and time (interval between biopsy and subsequent treatments)-dependent variability and a biopsy sample is just a snapshot of the tumor not reflecting the overall immune microenvironment [79]. Recent data revealed that multiple immune microenvironments may coexist in different metastasis within a single ovarian cancer patient [80]. In addition to intratumor heterogeneity, it should be kept in mind that the immune response is a very complex and dynamic process taking place in different sites other than TME, including lymph nodes and blood. Taken together, these data suggest that even if tumor PD-L1 expression facilitates enrichment with people who are most likely to respond to ICIs, it could not necessarily provide reliable information on the overall systemic immune status at single patient level. For this reason, the evaluation of PD-L1 status alone should not be used as negative predictor to exclude pretreated patients from ICIs, but should be always integrated with the evaluation of other potential biomarkers.

Potential biomarkers emerging from translational research

A deeper understanding of the cancer immunity cycle has allowed to elucidate the complex interactions between tumor cells and host immune system and to identify the multiple environmental factors that modulate intensity and timing of the antitumor immune response at single patient level [81], defining the so called ‘cancer immunity set-point’ [82], an individual immune profile which can predict response to ICIs. Beyond tumor PD-L1 expression, different clinical, pathological and molecular parameters have been explored in translational studies as predictive biomarkers for clinical use (Figure 3).

Clinical factors

In order to identify additional biomarkers to guide patients’ selection, multiple clinical determinants have been extensively searched. Poorer clinical prognostic factors, such as Eastern Cooperative Oncology Group Performance Status = 1, time since last treatment less than 3 months and progressive disease as best response to prior treatment, combined with no tumor PD-L1 expression, were initially associated with a significant higher risk of death within 3 months of treatment with nivolumab in the post hoc analysis of CheckMate 057 trial [72]. However, the same parameters were also observed in the majority of patients who were still alive after 3 months of therapy with nivolumab, hence indicating that actually we do not have reliable clinical parameters to predict clinical benefit. As

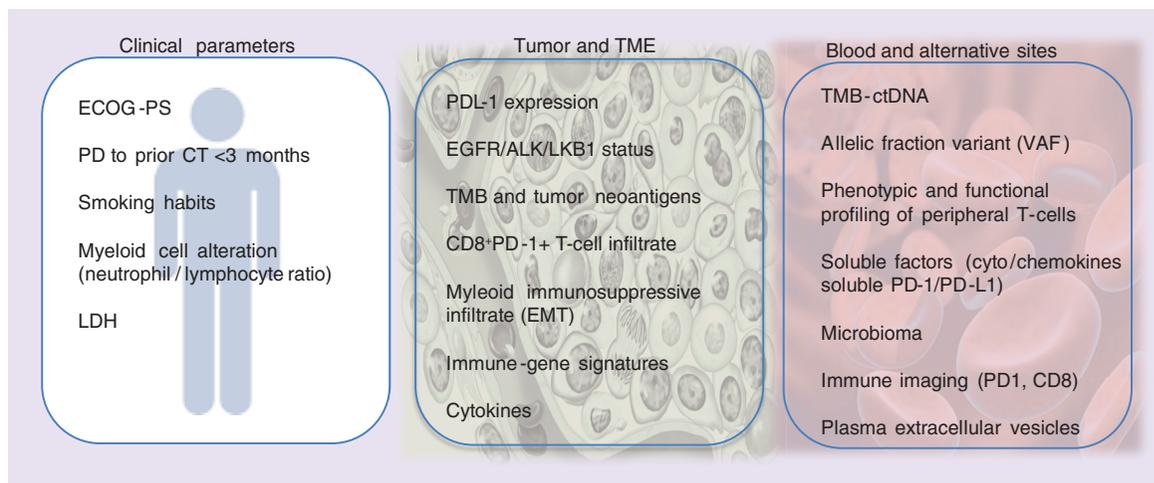


Figure 3. Predictive biomarkers to PD-1/PD-L1 blockade.

shown by randomized trials included in a recent meta-analysis ICI prolonged survival of ever-smokers patients with advanced NSCLC patients while failed to improve survival in never-smokers, suggesting smoking status as predictive marker for clinical use [83]. Rizvi *et al.* have recently reported that patients with NSCLC harboring a smoking-related molecular signature had higher mutational burden and increased clinical benefit from pembrolizumab, providing the biological rationale to this clinical observation [84]. An elevated pretreatment neutrophil-to-lymphocyte ratio (NLR) may be considered as a clinical biomarker associated with poor prognosis in lung cancer [85,86], while both high baseline and follow-up NLRs are associated with progressive disease and inferior survival in NSCLC patients [87]. Some studies investigated also the prognostic/predictive role of NLR in NSCLC patients receiving immunotherapy with nivolumab, overall confirming that high pretreatment NLRs are associated with inferior outcomes and early treatment failure, regardless of other prognostic factors [88,89], while absolute lymphocyte count should not be used as criteria to exclude patients from ICIs [90]. Recently, Mezquita *et al.* showed that combining NLR and lactate dehydrogenase (LDH) could represent a potentially useful tool to select patients unlikely to benefit from ICI treatment [24]. Overall, these data suggest that the myeloid fraction of peripheral blood cells plays an important role in modulating antitumor response and should be further investigated as predictive biomarker of ICIs activity.

Genetic alterations

Looking for 'molecular' predictors, preclinical data showed that both the EGFR and EML/ALK oncogenic signaling in NSCLC cells could directly induce PD-L1 expression, thus suggesting that treatment with PD-1 inhibitors could be more effective in EGFR-mutated tumor models [91,92]. However, the retrospective analysis of response rate by EGFR mutation status in all the four randomized studies comparing ICIs versus docetaxel in pretreated NSCLC patients [6–9], together with a recent meta-analysis [93], have consistently demonstrated that there was no survival difference between ICIs and chemotherapy in EGFR-mutant NSCLC patients who progressed to prior EGFR-tyrosine kinase inhibitors (TKI). These data point to hypothesize that patients with EGFR-mutated tumors may not be good candidates to therapy with ICIs. The discrepancy with preclinical data could stem from the very low tumor mutation burden (TMB) featuring EGFR-mutated tumors and the consequent reduced number of 'neo-antigens' triggering a protective immune response [94], as described more in details below. Hence, even if both EGFR-mutated and ALK-translocated tumors are often characterized by a significant oncogene-driven PD-L1 expression [95,96], the very low rate of associated TILs makes the chances that ICIs could trigger an effective immune response rather negligible. Recently, Gainor *et al.* showed that tumor PD-L1 overexpression and high TILs rate were simultaneously detected in only 1/57 TKI-naive, 5/57 TKI-resistant EGFR-mutated and none of ALK-positive NSCLC patients [97]. Interesting a recent paper published in *Annals of Oncology* [98] analyzing the survival outcomes of 25 EGFR-mutated NSCLC patients receiving nivolumab after EGFR-TKI, revealed that T790M-negative patients received major benefit from nivolumab as compared with the T790M positive subset, likely due to the loss of the oncogene signaling. Recent findings revealed that EGFR-mutated tumors are

associated with a high expression of the immunosuppressive molecule CD73 and reduced expression of IFN- γ mRNA signature suggesting that the overexpression of CD73 could be responsible for the reduced activity of ICI in this subset of NSCLC [99]. According to these evidences, the preliminary results of the Phase II ATLANTIC trial evaluating the clinical activity of durvalumab in heavily pretreated EGFR⁺/ALK⁺ NSCLC patients showed that some responses can occur also in oncogene addicted tumors who express high PD-L1 levels, however, a great and durable activity of ICI was limited to the subgroup of patients with high PD-L1 expression and EGFR/ALK wild-type [100]. Interestingly, the results of the Phase II BIRCH study have recently demonstrated a great activity of atezolizumab monotherapy as first-line treatment in PD-L1-positive NSCLC patients, regardless of tumor EGFR-mutation status [101], strengthening the role of PD-L1 in first-line but also suggesting that more efforts are needed to understand the complex mechanisms modulating the antitumor immune response in oncogene-addicted NSCLC.

Beyond EGFR and ALK, a recent meta-analysis investigated the predictive role of KRAS mutations in 519 patients with previously treated NSCLC included in the CheckMate 057, OAK and POPLAR randomized studies, suggesting a greater benefit in KRAS-mutant subgroups. However, this difference did not reach statistical significance and KRAS results were available only for 17% of patients, thus not allowing to recommend KRAS as predictive biomarkers for clinical use [93]. Several preclinical and clinical evidences showed that KRAS mutations are associated with a proimmunogenic TME and clinical responses to anti-PD-1 therapies. However, KRAS-mutant NSCLC is a heterogeneous disease, including different tumor subtypes with variable biological background and clinical response to immunotherapy. A recent work revealed that tumors with co-occurring KRAS/P53 mutations were associated with higher PD-L1 expression as well as elevated PD-L1⁺/CD8⁺ cell ratio and increased mutation burden as compared with both KRAS or P53 single mutation. Conversely, co-occurring inactivation of liver kinase B1 (LKB1)/serine threonine kinase 11 (STK11) tumor-suppressor gene was associated with lack of tumor response and shorter progression-free survival and OS as compared with LKB1/STK11 wild-type patients with KRAS-mutant lung adenocarcinoma, suggesting LKB1-loss as genomic biomarker of innate resistance to ICIs [102]. Other papers found that loss of phosphatase and tensin homolog (PTEN) and WNT/ β -catenin signaling pathway alterations also led to primary resistance to anti-PD-1 therapy in metastatic melanoma patients who received anti-PD-1 antibodies (nivolumab and pembrolizumab), those with PTEN present tumors acquired significantly larger response than those with PTEN absent tumors. Moreover, WNT/ β -catenin signaling pathway is involved in the primary resistance to checkpoint inhibitors. Spranger *et al.* showed in metastatic melanoma tissues that a higher CTNNB1 (β -catenin) score correlated with the lack of T-cells [103,104].

An additional mechanism of acquired resistance to ICI has been reported by Zaretsky *et al.* [105], who described that resistant melanoma patients may also display mutations in genes involved in the tumor sensitivity to immune response, such as, for instance, interferon-receptor-associated Janus kinase 1 and 2 (JAK1 and JAK2), or the antigen-presenting protein β -2-microglobulin. JAK1 and JAK2 mutations led to a lack of tumor response to IFN- γ , with loss of its antiproliferative effects on cancer cells, while the β -2-microglobulin mutation caused the loss of surface expression of major histocompatibility complex class I (HLA-I), with the consequent lack of recognition by tumor-specific T-cells [106].

Tumor mutational burden

Several pieces of evidence have recently revealed that a high TMB is significantly associated with an increased response to immunotherapy, favoring the creation of new foreign peptides defined as neoantigens that ultimately promote the immune recognition of cancer. A recent work classified lung cancer along with melanoma among the tumors with the highest mutation burden [107], likely due to the frequent mutagen exposure represented by tobacco smoke and UV lights, respectively. Rizvi *et al.* first demonstrated that an elevated nonsynonymous mutation burden, including DNA repair mutations, a molecular smoking signature and a high neoantigen load, was significantly associated with clinical activity of pembrolizumab in NSCLC patients [84]. After that several papers confirmed this observation [108,109], revealing that not all the generated neoantigens are equally immunogenic, being response to immunotherapy greater in presence of clonal than subclonal neoantigens and ultimately suggesting that targeting individual tumor neoantigens could represent an innovative approach to personalize cancer immunotherapy [110]. Recently, an exploratory analysis of the CheckMate 026 trial revealed a significant superiority of nivolumab over first-line platinum chemotherapy limited to the subgroup of patients with high TMB [111]. Particularly, the analysis of TMB revealed that patients whose tumors harbor both high TMB and PD-L1 more than 50% derived the maximum benefit from ICIs. Interestingly, a great benefit was also observed in the subgroup of patients with PD-L1 less than 50% and high TMB who would be currently excluded from ICI therapy, suggesting an additional

predictive role for clinical setting. Furthermore, it was recently demonstrated both the feasibility and reliability of TMB analysis from the blood of NSCLC patients who received ICIs, which would be particularly useful for about 30% of patients who lack tumor tissue for molecular analysis [112]. This retrospective analysis of both POPLAR and OAK studies revealed that a high-blood TMB significantly predicted progression-free survival of pretreated NSCLC patients receiving atezolizumab, providing very interesting evidences that, if prospectively validated, will offer a valid tool for oncologists to easily monitor the efficacy of ICIs therapy. However, recent data suggest that TMB could be considered as an imperfect surrogate biomarker not always reflecting the overall tumor neoantigen load, because different types of molecular aberrations other than genetic mutations determine the final tumor immunogenic phenotype [113], thus explaining while tumors with a low TMB also respond to ICIs.

Loss of mutation-associated antigens may lead to acquired resistance during immunotherapy. In mouse models, elimination of mutation-associated antigens by a specific T-cell could be a mechanism of cancer immunoeediting [114]. In melanoma patients who received adoptive T-cell transfer, the neoantigens recognized by T-cell are lost from tumor cells through immunoselective pressure [115]. Recent evidences showed that a potential mechanism of acquired resistance under PD-1/PD-L1 blockade therapy in initially responding NSCLC patients is represented by the loss or the reduced frequency of mutation-associated tumor neoantigens [116].

Tumor lymphocyte infiltrate

The positive prognostic value of intratumor CD8⁺ T-cells infiltrate in NSCLC has been known for several years [14]. Recent data indicate that the sensitivity to ICIs might rely on the presence of spontaneous CD8⁺ T-cell infiltrate expressing high levels of PD-1 and TIM3 (hot tumors) indicating the presence of a preexisting suppressed immune response that may be reactivated by anti-PD-1 agents, and ultimately suggesting an additional role as predictive biomarker. Conversely, resistance might be linked to reduced T-cell activation, defective tumor immunogenicity and a predominance of immunosuppressive cells, such as Tregs, MDSCs or secreted factors including TGF- β or inhibitory markers in the TME (cold tumors) [77]. Both Tregs and MDSCs activity can be stimulated by IDO, which is produced by cancer cells or immune cells [117,118]. IDO also affects the catabolism of tryptophan and produce immunosuppressive metabolites such as kynurenine, which inhibits the proliferation of T-cells and induces T-cell energy and apoptosis [119]. Combination of systemic inhibition of IDO and PD-1/PD-L1 blockade showed promising activity in early clinical trials in melanoma [120,121] and is currently under investigation also in lung cancer. Thommen *et al.* studied tumor-infiltrating CD8⁺ T-cells of 32 patients with NSCLC and revealed that the coexpression of PD-1, TIM3, LAG3, CTLA-4, and B and T lymphocyte attenuator was associated with resistance to anti-PD-1 therapy through severe defects in T-cells cytokine production, proliferation and migration [122]. An immune evasion to anti-PD-1 therapy has been studied by Koyama *et al.* [123] in two fully immunocompetent mouse models of lung adenocarcinoma progressing after response to anti-PD-1 therapy. The authors observed an upregulation of alternative immune checkpoints, notably TIM3, in PD-1 antibody-bound T-cells; moreover TIM3 with other inhibitory receptors including PD-1 and LAG3 mediated T-cell exhaustion. In tumors progressing after response to anti-PD-1 therapy, and with upregulation of TIM3, they demonstrated a survival advantage with addition of a TIM3-blocking antibody following failure of PD-1 blockade, identifying a therapeutic strategy that can prolong the efficacy of immunotherapy.

Tumeh *et al.* performed serial biopsies from melanoma patients treated with pembrolizumab demonstrating that not only the density but also the specific intratumor location of CD8⁺ T-cells infiltrate, as well as the simultaneous presence of high PD-L1 expression may influence the response to ICIs [54]. Subsequently, another work revealed that combining both TILs and IHC PD-L1 expression status allowed the identification of four different subgroups of TME in melanoma patients that could benefit from different treatment strategies, including combination therapies [124]. Similar findings have been also observed in NSCLC patients, where the combination of IHC PD-L1 expression and tumor-associated immune cells density identified different subsets of TME associated with distinct clinic-pathological variables and survival outcomes [125]. A recent study performed transcriptomic profiling of CD8⁺ T TILs in tumor tissue of treatment-naive lung cancer patients, revealing a wide heterogeneity in the expression of TCR and other immunotherapy targets, including PD-1, TIM3, LAG-3 and CTLA-4, which could guide the selection of different immunotherapy strategies for each patient [16]. Interestingly, this analysis identified a specific subset of enriched TILs expressing a memory cell phenotype, named as tissue-resident memory CD103⁺ cells, which seem to play a key role in the T-cell-mediated response and are associated with a better survival in lung cancer patients, regardless of CD8⁺ T-cells tumor density. Another study characterized the different infiltrating T-cell subsets activated in both preclinical and clinical melanoma samples under ICIs therapy by mass cytometry,

demonstrating that both anti-PD-1 and anti-CTLA-4 agents induce the expansion of a specific exhausted-like CD8⁺ T-cell population while the anti-CTLA-4 MoAbs favor also the expansion of specific ICOS⁺ Th1-like CD4⁺ T-cells, inducing Th1 CD4⁺ immune responses and ultimately feeding the pool of short-term memory T-cells that play a key role in ICIs-induced immune response and would be otherwise destined to run out [126]. Furthermore, it allowed to identify a new subset of hyperexhausted effector T-cells that seem to be not sensitive to single-agent PD-1-blockade and could likely benefit from combination strategies including CD28/B7 pathway costimulation [127]. These evidences elucidated the multiple mechanisms underlying ICIs response in the TME, thus providing the rationale for combination and personalized strategies in different tumor types, including lung cancer.

Immune-gene signatures & cytokines

Immune-gene signatures detected both in tumor tissue and in plasma of NSCLC patients receiving ICIs have been investigated as potential surrogate of individual immune response. Particularly in an exploratory analysis of the Phase II POPLAR trial, Fehrenbacher *et al.* analyzed the expression profile of immune-related genes at mRNA level, showing that patients with high expression of T-effector IFN- γ -associated genes received major benefit from atezolizumab [65]. Similarly, NSCLC patients with high IFN- γ mRNA signature in baseline tumor biopsies had greater response and longer survival to the anti-PD-L1 durvalumab, irrespectively of PD-L1 status [128]. Interestingly, a recent study in melanoma patients resistant to anti-PD-1 identified a cohort of 26 transcriptomic signatures, named 'IPRES' signature, including immunosuppressive genes (*IL10*, *VEGFA*, *VEGFC*), mesenchymal transition genes (*AXL*, *ROR2*, *WNT5A*, *LOXL2*, *TWIST2*, *TAGLN*, *FAP*), and monocyte and macrophage chemotactic genes (*CCL2*, *CCL7*, *CCL8* and *CCL13*) commonly associated with increased hypoxia, angiogenesis, mesenchymal transition and wound healing in different cancer types such as melanoma, kidney clear cell carcinoma, colon, lung and pancreatic adenocarcinoma [129]. Other studies found that increased VEGF secretion reduces the function of effector T-cells, decreases their presence into the tumors and correlates with higher PD-1 expression on CD8⁺ T-cells [130–132]. In patients with advanced NSCLC, the combination of nivolumab with paclitaxel/carboplatin/bevacizumab revealed a good antitumor activity [133] and several ongoing trials are currently investigating the synergism of antiangiogenic and immunotherapy treatments. The TME controls T-cells migration from the circulatory system into tumors by chemokines. Th1/Tc1 responses are IFN- γ -inducible by chemokines CXCL9, CXCL10 and CXCL11 that bind CXCR3 expressed on T lymphocytes. These chemokines mediate the angiogenesis inhibition, and promote Th1-mediated immunity, inducing tumor immune-mediated destruction by recruitment of CXCR3-expressing T and natural killer cells [134–136]. Low levels of CXCR3 ligands in TME reduce CD8⁺ T-cells infiltration that may affect the immunotherapy efficacy. A study by Peng *et al.* [137] showed that epigenetic silencing of Th1-type chemokines CXCL9 and CXCL10 inhibits T-cells homing. Recently, Borghaei *et al.* investigated the predictive role of the cytoscore, an integrated serum cytokine signature generated to quantify the effect of each identified cytokine set on OS in patients with both squamous and nonsquamous advanced NSCLC receiving nivolumab in the CheckMate 063/017/057 studies, showing that patients with a high serum cytoscore presented a significant longer OS (HR: 0.48; 95% CI: 0.36–0.64) [138].

Overall these reports showed that the immune signatures could be used as indirect indicators of the general individual immune status, helping clinicians to select patients with a preexisting immunity who are more likely to benefit from immunotherapy. However, they require further validation in prospective studies including larger cohorts of patients. In addition to these, several other potential biomarkers are currently under investigation in translational studies and are expected to be early added to the aforementioned list.

Peripheral blood biomarkers

Most of the predictive and resistance biomarkers that are currently under evaluation or validation refer to TME and thus have to face the obvious hurdles of tumor biopsy availability and heterogeneity. As a consequence, remarkable efforts are ongoing to identify selection factors through alternative routes, relying, for instance, on blood samples, microbioma or *in vivo* imaging. Blood is being assessed in the context of a liquid biopsy approach, to detect tumor DNA mutations that could surrogate the role of mutational burden as source of immunogenic neoantigens. Recent studies evaluated circulating tumor (ct)DNA level changes by determining the allelic fraction variant before and after four cycles of immunotherapy showing that early ctDNA level changes could predict response/resistance to ICIs [139]. In addition to that the total number of molecular alterations detected in ctDNA could be also used to determine a 'hypermutated state' and predict immunotherapy efficacy [140].

The presence of circulating T-cells and antibodies specific for tumor antigens – signs of an active cellular and humoral antitumor systemic immunity – could also provide predictive information of patients' responsiveness. This is indicated by preliminary data showing that the level of SOX-2 (a transcriptional factor overexpressed in lung cancer)-specific T lymphocytes and antibodies identifies NSCLC patients responding to treatment with nivolumab [141]. Comparably, an early PD-1⁺ CD8 T-cell response within 4 weeks of treatment has been observed in 80% of patients with clinical benefit to anti-PD-1 therapies, while about 70% of patients with disease progression had either a delayed or absent PD-1⁺ CD8 T-cell response [127]. Circulating myeloid cells are also being analyzed to assess systemic immunosuppressive and inflammatory state that is expected to hamper response to immunotherapy. Despite these correlations were not always confirmed in additional studies, it can be foreseen that tumor-related T-cells and immunosuppressive responses represent a promising source of future biomarkers, given the systemic nature of the interactions that cancer cells engage with our immune defense.

Microbioma

Increasing evidence suggests that the gut microbiota promotes antitumor immunity and facilitates anti-PD-L1 efficacy. Two studies in mice showed that certain intestinal bacteria can promote antitumor immune responses and facilitate responses to immune checkpoint blockade. Sivan *et al.* investigated the effects of the microbiome on responses to PD-1 pathway blockade. The authors studied melanoma growth in mice with distinct commensal microbiota and analyzed differences in antitumor immunity. They observed that Bifidobacterium was present in mice responders to immunotherapy, and that oral Bifidobacterium administration alone led to tumor control, while in combination with anti PD-L1 therapy inhibited tumor growth. They showed how cancer immunotherapy response can be modulated by changing the microbiota, and that Bifidobacterium species improve DC activation and better antigen presentation with activation of CD8⁺ T-cells [142].

Vétizou *et al.* studied the interaction between microbiome and responses to CTLA-4 blockade. In mice bearing MCA205 sarcomas treated with CTLA-4 blockade, the analyses of fecal samples revealed that treatment reduced the levels of Bacteroidales and Burkholderiales and increased the levels of Clostridiales populations. In mice and patients, T-cell response and efficacy of CTLA-4 blockade were related specifically to *B. Thetaiotaomicron* or *B. fragilis*. Germ-free mice or those treated with antibiotics did not respond to CTLA-4 blockade, while germ-free mice receiving fecal transplants from patient responders to anti-CTLA-4 treatment had marked tumor responses after anti-CTLA-4 treatment and showed colonization by *B. fragilis*. This study showed the relevant role of Bacteroidales in the immunostimulatory effects of CTLA-4 blockade [143]. These data are interesting because of the prospect that administering a probiotic may improve antitumor immune responses during immune checkpoint blockade [144].

Immune imaging

The object of numerous researches in the field of immunocyte inhibitors is immunoimaging, in other words, the ability to visualize the distribution of immune cells on the human body *in vivo* through the corresponding antibodies labeled with radioactive substances that are captured by the positron emission tomography (PET) machinery to then compose tomographic images.

All these help analyze the enormous heterogeneity of tumor and immune systems and the actual distribution of immune cells involved in the various parts of the body, photographing what is the expression of the immune response *in vivo*.

Therefore, it is attempting to define a quantitative biomarker that can assist in the selection and treatment of patients who undergo such therapies, modulating treatment dosage and effectiveness through the dynamic and noninvasive visualization of the immune response to the drugs in question [145–147].

Murine specimens have recently been the object of the anti-PD-1 immunoblot inhibitor nivolumab, which was radiolabeled with Zirconium-89. After administration, it was possible to outline the biodistribution of tumor-expressing PD-1 tumor cells on various body sites by PET, which was subsequently verified by histological analysis [147,148].

Another study in murine models, however, used another ipilimumab immunocyte inhibitor that was labeled with ⁶⁴Cu-DOTA and CTLA-4 expression biodistribution was also detected, histologically confirmed, showing enhanced and persistent accumulation in CTLA-4-expressing tissues. Therefore, the use of a noninvasive method as the immune-PET may have an important value to assess patients' response to immunotherapy by longitudinal noninvasive monitoring of CD8⁺ T-cells [149].

How to contribute to the validation of novel predictive factors: a new era for spontaneous clinical trial

The clinical development of ICIs introduced an exciting antitumor immunotherapy era. Long-term responses have been observed in multiple cancer types. Even if PD-1 or PD-L1 inhibitor monotherapy is largely well tolerated, the risk of adverse events associated with immunization increases with the use of combination regimens. Therefore, the identification of predictive biomarkers represents an urgent challenge to optimize patients' benefit, minimize the risk of toxicity and guide the combined approaches. The spotlight has been almost always on PD-L1 expression on cancer cells. Although PD-L1 positivity has been translated into a clinical benefit for this population slice, PD-L1 testing alone is insufficient for patients' selection in most cancer types, including NSCLC. There are several problems that are still pending regarding the diagnostic reliability of PD-L1 testing on cytological and small biopsy samples or liquid biopsy, tumor heterogeneity biomarkers, resistance mechanisms in positive PD-L1 tumors and responsiveness of PD-L1-negative tumor; rebiopsy to determine PD-L1 expression; heterogeneity of PD-L1 expression in primary tumor as compared with metastatic sites or after treatments. In this review, we explored emerging data on new biomarker strategies. As discussed above, there are a number of peripheral and tumor-based laboratory tests that have been screened as potential biomarkers to predict immunotherapy efficacy. Unfortunately, although some of these have been associated with positive outcomes, no one has been prospectively validated yet to allow patients' selection in clinical setting. A lot of efforts are in place to find biomarkers with satisfying predictive value that can help clinicians to select those patients who will most likely benefit from the blockade of immunological checkpoints and will get durable PD-1/PD-L1 inhibition benefits, and save resistant patients from an ineffective therapy and treatment-related toxicity. The refinement of existing biomarkers and the identification of new predictors will be key to ensuring the effective and safe use of these agents, such as PD-1/PD-L1.

The goal we have set as a group of young colleagues who mainly deal with thoracic disease in the 'immunotherapy era,' is promoting spontaneous translational studies characterized by a systematic collection of clinical data, tumor tissues and peripheral blood samples from NSCLC patients receiving ICIs at various medical centers for the identification of immune-related biomarkers. This analysis could also include patients' stool samples for the setup of the microbiota, which, as discussed above, is involved in immune surveillance and is known to influence immune system maturation and activity. An optimal sampling timing should always include a baseline collection before the patient undergoes any treatment; a second evaluation during the early months of therapy; and a last at the time of disease progression. This process would allow to obtain real-time and *in vivo* information on the treatment efficacy/resistance in NSCLC patients during the course of ICIs, providing evidence-based answers to the aforementioned clinical questions.

Conclusion

A deeper understanding of the immune landscape of cancer, including immune-evasion strategies, have led to breakthrough therapeutic advances for patients with NSCLC and have created a platform for future therapeutic developments. Immune-checkpoint inhibitors targeting PD-1/PD-L1 axis represent the current gold standard for pre-treated patients, and are now moving to first-line treatment of advanced NSCLC. With the current emphasis on precision medicine and biomarker-driven drug development, identifying predictive biomarkers remain a crucial issue in order to select those patients who may gain most benefit from immunotherapy or combination strategies.

Future perspective

Since the immune response is a very complex and dynamic spatiotemporal process, only the combination of different biological parameters integrating information from the tumor genomics and microenvironment, individual immune system and peripheral blood compartment will allow to detect the immunological profile at single patient level defining the 'cancer immunogram' [150]. Recently, Karasaki *et al.* performed whole exome and RNA sequencing in 20 patients with advanced NSCLC, identifying three different immunogram patterns. The 'T-cell rich' pattern, characterized by gene signatures of immune cell and checkpoint molecules, suggesting a preexisting antitumor immunity; and the T-cell poor pattern, characterized by a lack of immune cells and an intermediate pattern [151]. Similarly, Chen and Melmann described three basic immune phenotypes associated with clinical response to ICIs: the inflamed phenotype, characterized by the presence of abundant immune cells, high checkpoint protein expression and proinflammatory cytokines in the tumor, likely to respond to immunotherapy; the immune-excluded phenotype, characterized also by the presence of abundant immune cells that are confined to the stroma and do not penetrate inside the tumor; and the immune-desert phenotype characterized by paucity of immune cells both

in the tumor and in the stroma, and presence of regulatory cells and cytokines associated with immune suppression or tolerance. Both immune-excluded and desert phenotypes may be considered as noninflamed profiles, suggesting the absence of a preexisting antitumor immunity, and rarely respond to ICIs [82]. The evaluation of patients' immunological profile at different time points during the course of ICIs therapy would allow to build the cancer immunogram as model of integrated biomarkers and ultimately overcome biological heterogeneity and personalize immunotherapy strategies. We are at the beginning of a creative period of bottom-up research activity, organized through pilot projects of increasing scope and scale, from which best practices will progressively emerge. As young oncologists, fighting lung cancer we would like to highlight that the broad current use of ICIs in clinical practice offers the unique opportunity of actively contributing to this translational research in NSCLC patients to build a bridge between research and clinical practice.

Executive summary

- In the past decade, intense research on cancer immunology allowed to elucidate the molecular basis of cancer immunogenicity and immune-editing process.
- The PD-1/PD-L1 immune checkpoint emerged as a key regulator of antitumor immune response in cancer patients.
- Systemic immunomodulatory drugs targeting the PD-1/PD-L1 axis were able to reactivate host responses and to mediate outstanding clinical benefit in a sizable proportion of patients with non-small-cell lung cancer (NSCLC).
- Immune checkpoint inhibitors, nivolumab, pembrolizumab and atezolizumab are now approved as standard treatment options in patients with advanced NSCLC.
- With the current emphasis on precision medicine, identifying predictive biomarkers remains a crucial issue in order to select the subset of patients who benefit most from immunotherapy.
- The expression PD-L1 on tumor cells should not be used as negative predictor to exclude pretreated patients from ICIs; while use of pembrolizumab in first line is currently limited to NSCLC patients with tumor PD-L1 expression more than 50%.
- Beyond tumor PD-L1 expression, tumor mutational burden, tumor genetic alterations, tumor lymphocyte infiltrate, inflammatory signatures and cytokines, microbioma, immune imaging and peripheral blood markers are currently investigated as predictive biomarkers for clinical use in translational research studies.
- The combination of different biological parameters integrating information from the tumor genomics and microenvironment, individual immune system and peripheral blood compartment will likely allow to define the 'cancer immunogram' at single patient level.
- The broad current use of immunotherapy in clinical practice offers the unique opportunity of actively contributing to this translational research in NSCLC patients to build a bridge between research and clinical practice.

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