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Editors: Baohui Han
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Lung Cancer Immunotherapy: PD-1/PD-L1 Focused (FIRST EDITION)

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We are pleased to announce that the "AME Research Time Medical Book Series" launched by AME Publishing Company has been published as scheduled.

Finishing my medical degree after 4 years and 3 months of study, I decided to leave the path toward becoming a doctor only after 3 months of training. After that, I had been muddling through days and nights until I started engaging in medical academic publishing. Even 10 years after graduation, I had not totally lost the affection for being a doctor. Occasionally, that subconscious feeling would inadvertently arise from the bottom of my heart.

In April 2011, Mr. Tiantian Li, the founder of DXY.cn, and I had a business trip to Philadelphia, where we visited the Mütter Museum. As part of The College of Physicians of Philadelphia, the museum was founded in 1858 and has now become an exhibition hall of various diseases, injuries, deformities, as well as ancient medical instruments and the development of biology. It displays more than 20,000 pieces of items including pictures of wounded bodies at sites of battle, remains of conjoined twins, skeletons of dwarfs, and colons with pathological changes. They even exhibited several exclusive collections such as a soap-like female body and the skull of a two-headed child. This museum is widely known as "BIRTHPLACE OF AMERICAN MEDICINE". Entering an auditorium, we were told by the guide that the inauguration ceremony of the Perelman School of Medicine at the University of Pennsylvania would take place there every year. I asked Mr. Li, "If it was at this auditorium that you had the inauguration ceremony, would you give up being a doctor?" "No," he answered.

In May 2013, we attended a meeting of the British Medical Journal (BMJ) and afterwards a gala dinner was held to present awards to a number of outstanding medical teams. The event was hosted annually by the Editor-in-Chief of the BMJ and a famous BBC host. Surprisingly, during the award presentation, the speeches made by the BMJ never mentioned any high impact papers the teams had published in any prestigious journals over the past years. Instead, they laid emphasis on the contributions they had made on improving medical services in certain fields, alleviating the suffering of patients, and reducing the medical expenses.

Many friends of mine wondered what AME means.

AME is an acronym of "Academic Made Easy, Excellent and Enthusiastic". On September 3, 2014, I posted three pictures to social media feeds and asked my friends to select their favourite version of the AME promotional leaflet. Unexpectedly, we obtained a perfect translation of "AME" from Dr. Yaxing Shen, Department of Thoracic Surgery, Zhongshan Hospital, Shanghai, who wrote: enjoy a grander sight by devoting to academia (in Chinese, it was adapted from the verse of a famous Chinese poem).

AME is a young company with a pure dream. While having a clear focus on research, we have been adhering to the core value of "Patients come first". On April 24, 2014, we developed a public account on WeChat (a popular Chinese social media platform) and named it "Research Time". With a passion for clinical work, scientific research and the stories of science, "Research Time" disseminates cutting-edge breakthroughs in scientific research, provides moment-to-moment coverage of academic activities and shares little-known behind-the-scene stories. With global vision, together we keep abreast of the advances in clinical research; together we meet and join our hands at the Research Time. We are committed to continue developing the AME platform to aid in the continual forward development and dissemination of medical science.

It is said that how one tastes wine indicates one's personality. We would say how one reads gives a better insight to it. The "AME Research Time Medical Books Series" brings together clinical work, scientific research and humanism. Like making a fine dinner, we hope to cook the most delicate cuisine with all the great tastes and aromas that everyone will enjoy.

Stephen Wang
Founder & CEO,
AME Publishing Company

The recent years have witnessed at least two tremendous steps forward in the war against lung cancer: the identification of oncogene-addiction as a key to precisely target an extremely featured population of patients and the 'renaissance' of immunotherapy, which, after years of none or moderate clinical advances, has significantly revolutioned the way to unblock and trigger the innate immune response. Both innovations allow clinicians to offer to patients affected by such disease a life-expectancy which nowadays in many cases differs from what the very same doctors have experienced and they have been used to discuss with patients and families no longer than 5-10 years ago.

Why that happens this way and now? On one hand, brilliant and brave investigators significantly contributed to science by characterizing human and cancer genomes, tracking those bio-molecular pathways which have been demonstrated to dysregulate normal cells' homeostasis and to drive tumor transformation and proliferation; concurrently, a series of smart scientists focused upon what did happen around tumor cells and how the surrounding microenvironment interplay with such pathological process with the final result to not reject that.

In this regard, the discovery of the interaction between PD1 and PD-L1 at the immune-checkpoint as the key-block inducing immune cancer tolerance, indicated to develop monoclonal antibodies specifically engineered to disrupt this binding, thus allowing immune reaction cascade to start and face the tumor. From the clinical standpoint, these antibodies have demonstrated to work in a series of tumors and settings where previously no major advances were reported in the latest 15 years, such as non-small-cell lung cancer, with a particular regard to squamous histology.

A critical finding of this way to unravel the tumor immune escape was that the benefit of these drugs did not significantly impact upon traditional intermediate end-points (such as progression-free-survival, response rate), while the overall survival was significantly improved, with a characteristic long-term effect. In addition, the unique biomarker able to maximize the benefit of antibodies targeting PD1/PD-L1 was PD-L1 overexpression, although it did not entirely allow to identify all potentially sensitive patients (i.e. a series of negative patients did respond to immunotherapy as well). As expected, translational and basic research with innovative technologies has arrived to help to solve this crucial issue, by suggesting a potential role of other key-players in activating immune response, such as the mutational and neoantigen load, which both represent today a new research frontier with fascinating implications.

All these reasons leave to clinical and translational research a series of major challenge for the forthcoming years, such as the optimization of PD-L1 as a selection factor, the identification of additional biomarkers beyond PD-L1 (mutational and neoantigen load), the development of more reliable preclinical model able to specifically derive hypotheses for clinical research for drugs targeting the tumor-host microenvironment, and the development of clinical trials plans with methodological tools specifically tailored to how immunotherapy impact upon the patients' prognosis.

With this intent, I am sincerely honoured to serve as a Co-Editor of this special issue, whereas international experts and opinion leaders in lung cancer research who are dedicating their career to clinical and translational research have been asked to report their personal views and perspectives on a series of given topic according to their skilled expertise. What clearly emerges for readers is a model of team-work, where the collaboration as a network of different professionals (such as basic and translational scientists, pathologists, immunologists, clinicians, methodologists) may pave the way to further improve clinical opportunities for lung cancer patients in the very near future.

Emilio Bria

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Immunotherapy for lung cancer: Its time has finally come

There has never been such an exciting time to be an oncologist and investigator in lung cancer. Even as recently as the early 2000s, systemic therapy for lung cancer still relied on combinations of cisplatin-based chemotherapy, which were associated with moderate or severe toxicity in up to 50% of cases and dismal 5-year overall survival rates of only about 1% to 5% for patients with stage IV disease. Over the past decade, image-guided radiotherapy, particularly intensity-modulated radiotherapy (IMRT) and stereotactic ablative radiotherapy (SABR), has fundamentally improved the ability to precisely target tumors even in complex anatomic locations, which has led to reduced toxicity, increased local/regional control, and even improved survival for patients with early-stage or locally advanced lung cancer. However, distant metastasis remains the dominant pattern of failure after definitive local therapy. In addition, even now most patients are found to have stage IV disease when the lung cancer is diagnosed. Over a similar period, the advent of personalized molecular targeted therapy has significantly improved rates of response and overall survival in the roughly 20% to 30% of patients with metastatic lung cancer that harbors mutations in the genes for EGFR, ALK, ROS-1, and BRAF. But what about the rest of the patients, those without targetable gene mutations?

Immunotherapy was not considered an option for lung cancer treatment until about 2015, when PD-1 checkpoint immunotherapy was found to improve progression-free survival and, later on, overall survival rates (up to 16% at 5 years) with fewer and much less severe side effects than second-line chemotherapy for patients with stage IV non-small cell lung cancer (NSCLC) that did not respond to conventional first line chemotherapy. Amazingly, in a very brief time immunotherapy has moved from second-line to first-line therapy for stage IV squamous or non-squamous cell lung carcinoma, being given as PD-1 immunotherapy alone, or in combination with CTLA4 immunotherapy, or in combination with conventional chemotherapy or angiogenesis inhibitors depending on the tumor's PD-L1 status, total mutation burden, and the presence of other molecular markers. After the June 2018 meeting of the American Society of Clinical Oncology, conventional chemotherapy is being edged over to second-line therapy, or as an adjunct to immunotherapy, for many patients with lung cancer. Indeed, the US National Comprehensive Cancer Network modified its guidelines several times in 2017 and again in 2018 based on the emerging scientific evidence in support of immunotherapy.

By the end of 2017, adjuvant PD-L1 immunotherapy was found to improve progression-free survival for patients with stage III NSCLC treated with concurrent chemo-radiotherapy, and subsequently a benefit in terms of overall survival was reported as well. This is the first time in the past decade that overall survival has significantly improved for patients with stage III NSCLC, as demonstrated in a prospective randomized study that included radiotherapy as definitive local therapy. Indeed, in various combinations with radiotherapy, surgery, and chemotherapy, immunotherapy has shown promising early results for both early and locally advanced lung cancer.

However, at this time the response rates of unselected patients with lung cancer to PD-1/PD-L1 immunotherapy alone remain low at 20% to 30%, underscoring the ongoing need to optimize immunotherapy for individual patients based on novel molecular markers. Moreover, combining immunotherapy with other forms of treatment such as chemotherapy, radiotherapy, or both may well overcome the development of resistance to immunotherapy and improve the therapeutic ratio. This has been a most exciting time for clinical research on this topic. The concept of combining immunotherapy with stereotactic ablative radiotherapy (I-SABR) has triggered extensive interest, and hundreds of preclinical and clinical studies are now ongoing. I-SABR and other strategies for optimizing immuno-radiotherapy are also being proposed in 2018. There is no doubt that tomorrow's lung cancer patients will be better treated than today's. This book provides unique, up-to-date information about the paradigm shift in biology and technology prompted by the advent of immunotherapy. More is coming. Stay tuned.

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Abstract: A deeper understanding of the key role of the immune system in regulating tumor growth and progression has led to the development of a number of immunotherapies, including cancer vaccines and immune checkpoint inhibitors. Immune checkpoint inhibitors target molecular pathways involved in immunosuppression, such as cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and programmed cell death protein-1 (PD-1)/programmed cell death ligand-1 (PD-L1) pathway, with the goal to enhance the host's own immune anticancer response. In phase I–III trials, anti-PD-1/PD-L1 antibodies have demonstrated to be effective treatment strategies by inducing significant durable tumor responses, with manageable toxicities, in patients with various malignancies, including those traditionally considered non-immunogenic, such as non-small cell lung cancer (NSCLC). Identification of predictive biomarkers to select patients for immune therapies is currently being investigated to improve their therapeutic efficacy. Transforming growth factor-β (TGF-β), a pleiotropic cytokine with immunosuppressive effects on multiple cell types of the innate and adaptive immune system, has emerged as one of the potential key factors modulating response to immune checkpoint inhibitors. However, due to the complexity of the anti-cancer immune response, the predictive value of many other factors related to cancer cells or tumor microenvironment needs to be further explored.

Keywords: Non-small cell lung cancer (NSCLC); programmed cell death protein-1 (PD-1)/programmed cell death ligand-1 (PD-L1); immunotherapy; transforming growth factor-β (TGF-β)

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Introduction

Non-small cell lung cancer (NSCLC) accounts for nearly 85% of all lung cancer cases and is commonly diagnosed at an advanced stage of disease. Even those patients undergoing potentially curative surgery can experience a recurrence, including systemic relapse, within few years, suggesting the systemic nature of the disease also in those patients with seemingly localized NSCLC (1,2). Different

cytotoxic chemotherapy regimens are currently used to treat advanced NSCLC patients, but they only contribute with modest improvements in survival. During the last few years, the use of molecularly targeted agents, has dramatically improved the prognosis of lung cancer patients harboring specific oncogenic alterations, including *EGFR* mutations and *ALK* rearrangement. However, oncogene-directed therapies are currently used in the clinical setting only for relatively small subgroups of patients, mainly with adenocarcinoma histology. Furthermore, despite initial significant clinical benefit from EGFR- or ALK-tyrosine kinase inhibitors, patients will inevitably progress within 1–2 years, due to development of acquired resistance (3,4). Thus, additional treatment strategies that could obtain long lasting disease control without increasing toxicity are still needed. In recent years, further understanding of the interaction between the immune system and tumor growth has led to the development of several immunotherapies, with the goal to boost the host's own immune anticancer response. These immunotherapies include immune checkpoint inhibitors, such as monoclonal antibodies directed against cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and programmed cell death protein-1 (PD-1)/programmed cell death ligand-1 (PD-L1) pathway, which have demonstrated therapeutic efficacy in a variety of human malignancies, including those historically considered as non-immunogenic, including lung cancer (5-7).

Immune response and cancer

Cancer cells harbor different genetic and epigenetic alterations; thus, a number of antigens that are potentially recognized and eliminated by the immune system are commonly expressed by tumors. Thymus-derived lymphocytes (T lymphocytes, T cells) activation and expansion are necessary for an effective adaptive immune response. Particularly, the main anti-tumor immune effector cells are represented by interferon-γ (IFN-γ)-secreting T cells, which are able to inhibit and kill malignant cells, thus impeding tumor growth and spread of the disease. Spontaneous lymphocytic infiltration is frequently observed in a variety of human cancers and in numerous studies tumor infiltrating lymphocytes (TILs) have been correlated with a more favorable clinical outcome of patients and also with response to treatment, including chemotherapy and immunotherapy (8-13). This can be explained by the fact that a component of this T-cell infiltrate is represented by tumor antigen-specific T cells activated in response to the growing tumors which exert their effector functions to eliminate cancer cells. However, in this model of T-cell infiltrated tumors, these cells subsequently become functionally inhibited by the effects of PD-L1 and indoleamine-2,3-dioxygenase (IDO) expression on tumor cells, driven by IFN-γ, and by the activity of T-regulatory (Treg) cells, thus contributing to immune escape (14). Immunologic responses are initiated when the antigens, presented by antigen presenting cells (APCs) in peptides complexed with major histocompatibility (MHC) complexes, are recognized by the T-cell receptor (TCR). Dendritic cells (DCs) are the most powerful APCs that migrate to lymph nodes after contact with tumor antigens and activate a tumor-specific-T-cell response (15). However, this first signal is not sufficient for activation of naïve T-cells. Additional co-stimulatory signals are required and are provided by the binding of CD28 on the T-cell surface with specific molecules, B7-1 (CD80) and B7-2 (CD86), on the APC (16). Once the T-cells are activated, the immune response enters the effector phase and T cells are capable of recognizing and destroying antigen-expressing tumor cells. The efficacy and duration of T-cell response depends on the balance between co-stimulatory and inhibitory signals that are delivered by different T-cell surface receptors. Immune co-stimulatory molecules include CD28, CD137, glucocorticoid-induced tumor necrosis factor (TNF) receptor (GITR), OX-40 and inducible costimulator (ICOS). Negative regulatory molecules or immune checkpoint molecules prevent overstimulation of immune responses and include cytotoxic T-lymphocyte antigen-4 (CTLA-4) and PD-1. These receptors interact with specific ligands of the B7 family: B7-1 (CD80) and B7-2 (CD86), that are present on APCs, but also on tumor cells. Immune checkpoints refer to molecules of inhibitory pathways that are crucial for maintaining self-tolerance and regulating the duration and amplitude of physiological immune responses against pathogens in periphery in order to avoid or minimize collateral tissue damage and inhibit chronic inflammation. CTLA4 and PD-1 represent the best characterized immune checkpoint receptors which deliver T-cell inhibitory signals (5,17). Inhibitory ligands are commonly overexpressed in APCs, tumor cells or other cells of the tumor microenvironment. Unfortunately, tumor cells can use these immune checkpoints as a defence mechanism. Indeed, it is well recognized that tumors, including lung cancers, are able to escape from immunosurveillance and maintain an immunosuppressive microenvironment through multiple mechanisms (18,19). These mechanisms include

recruitment of regulatory cells (e.g., Treg cells, myeloid-derived suppression cells, and type 2 macrophages), production of molecules suppressing antitumor T-cell responses [e.g., interleukin-10 (IL-10), indoleamine 2,3-dioxygenase, transforming growth factor-β (TGF-β)], low antigen presentation and immunomodulation of T-cell response through down-regulation of co-stimulatory molecules or enhancement of co-inhibitory molecules (immune checkpoints) on T cells, other immune cells and tumor cells.

The role of immune checkpoint molecules

CTLA-4 is expressed exclusively on T-cells and shares identical ligands (CD80 and CD86) on APC with the T-cell co-stimulatory receptor CD28. When the TCR is engaged by a cognate antigen, CD28 binds CD80/CD86 and induces T-cell activation. However, CTLA-4 has a much higher overall affinity for both ligands and inhibits the activation of T-cells by competing with CD28 in binding CD80 and CD86 (5,20,21). CTLA-4 regulates the early stage of T-cell activation through additional mechanisms, such as recruitment and activation of the Src homology region 2 domain-containing phosphatase-2 (SHP2) and protein phosphatase 2A (PP2A) via the YVKM motif in its cytoplasmatic domain. This results in attenuation of kinase signaling, such as PI3K/AKT pathway, induced by TCR and CD28 (22-25). CTLA-4 primarily regulates CD4+ T cells by downregulating the activity of T helper cells and enhancing the immunosuppressive activity of Treg cells.

PD-1 is an immune checkpoint receptor expressed by activated T cells as well as B cells and natural killer (NK) cells. Similarly to CTLA4, PD-1 is highly expressed on Treg cells and may enhance their proliferation. Unlike CTLA-4, PD-1 primarily inhibits T-cell activity in the effector phase within peripheral tissues and tumors (5,26-30). Upon antigen recognition, activated T cells upregulate PD-1 expression on their surface. Specifically, PD-1 is expressed on a large proportion of TILs from many cancers. PD-1 signaling involves binding to specific ligands, including PD-L1 (or B7-H1), PD-L2 (or B7-DC) (31,32). PD-L1 is expressed on macrophages and can be induced by inflammatory cytokines, mainly IFN-γ on different cell types, including cancer cells, epithelial cells of various tissues, lymphoid cells and myeloid cells. Numerous tumor types express PD-L1, including NSCLC, suggesting that this pathway is activated in different cancers to contribute to anti-tumor immune evasion. Structurally, PD-1 has a cytoplasmatic Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) as well as an Immunoreceptor Tyrosine-based Switch Motif (ITSM) and has been found to be capable of recruiting the phosphatases SHP-1 and SHP-2 (33,34). Specifically, after binding to the ligand, PD-1 is able to recruit SHP-2 to the ITIM domain, resulting in inhibition of downstream TCR and CD28 signaling, mainly PI3K/AKT pathway activation. The effects of PD-1/PD-L1 interaction are inhibition of T-cell proliferation, survival and effector functions (cytokine release and cytotoxicity), induction of apoptosis of tumor-specific T-cell and promotion of differentiation of CD4+ T-cells into Tregs. Excessive induction of PD-1 on T cells in the setting of chronic antigen exposure can induce an exhausted or anergic state in T cells, as demonstrated in PD-1 expressing TILs.

Other immune checkpoint molecules with potential relevant roles in anti-tumor immune response have been characterized. These include T-cell Immunoglobulin and Mucin domain-containing protein 3 (TIM3; also known as HAVCR2), lymphocyte activation gene-3 (LAG-3) and V-domain immunoglobulin suppressor of T-cell activation (VISTA) (17).

Inhibition of the immune checkpoint pathways has been shown to reverse cancer immunosuppression and activate T cells, thus enhancing anticancer immune responses.

PD-1/PD-L1 pathway inhibitors in lung cancer

Different monoclonal antibodies targeting either PD-1 or PD-L1 are currently in advanced phases of clinical development in patients with different tumor types, including lung cancer.

Anti-PD-1 drugs

Nivolumab is a fully human IgG4 monoclonal antibody targeting PD-1. Nivolumab disrupts negative signaling triggered by PD-1 binding to PD-L1 or PD-L2 and restores T-cell antitumor function. PD-1 is expressed on T cells as well as on many other immunologic cells, including B cells and NK cells, and therapeutic blockade of the PD-1 pathway may also influence the function of these cells (6). In a phase I nivolumab study, response rates were 18% in NSCLC, 28% in melanoma, and 27% in renal cell cancer. The drug was well-tolerated, with grade 3 or 4 adverse events observed in 14% of patients (35). Expansion cohorts of patients with heavily pretreated NSCLC demonstrated a response rate of 17%, with median duration of response of

17 months. Responses were rapid, with 50% of responders detected at the first response evaluation (8 weeks after starting treatment). Response rates were similar in squamous and non-squamous NSCLC. Median progression-free survival (PFS) for responders was 20.6 months. Median overall survival (OS) was 9.9 months. Survival rates at 1-, 2- and 3-year were 42%, 24% and 18% respectively (36). Common side effects were fatigue, anorexia, and diarrhea (similar in frequency to the overall study population); grade 3–4 toxicity was found in 14% and pneumonitis in 7% of NSCLC patients (3% had grade 3/4 pneumonitis). Three treatment-related deaths occurred among patients with NSCLC, each associated with pneumonitis.

In a phase II trial enrolling patients with advanced squamous NSCLC who had received two or more prior treatments, nivolumab was associated with a 14.5% response rate after an 11-month follow-up, with 3.3-month median time to onset of response (37). Responses were durable, with 77% of responders presenting ongoing responses during the analysis. Some of these patients (59%) had durable responses of 6 months or longer. Phase III trials in NSCLC have now been completed and results have been reported. The phase III CheckMate 017 trial was stopped early in January 2015 following an assessment conducted by the independent Data Monitoring Committee demonstrating a superior 3-month OS of nivolumab compared with docetaxel in patients with advanced squamous NSCLC pretreated with platinum-based chemotherapy (38). The median OS was 9.2 months with nivolumab *vs.* 6.0 months with docetaxel [hazard ratio (HR), 0.59; 95% CI, 0.44 to 0.79; P<0.001]. The response rate was 20% with nivolumab *vs.* 9% with docetaxel (P=0.008) and the median PFS was 3.5 months with nivolumab *vs.* 2.8 months with docetaxel (HR, 0.62; 95% CI, 0.47 to 0.81; P<0.001). The toxicity profile of nivolumab was more favourable than docetaxel. Benefit was independent of PD-1 expression. Recently, according to the results of the CheckMate 057 trial, nivolumab has been demonstrated to be the first PD-1 inhibitor to significantly improve OS in comparison with docetaxel (12.2 *vs.* 9.4 months; HR 0.73; 95% CI, 0.59 to 0.89; P=0.002), in previously treated patients with advanced non-squamous NSCLC with 27% reduction in risk of death and significantly improved overall response rate. In this study, tumor PD-L1 expression was found to be predictive of nivolumab benefit (39). Other studies are ongoing, including the phase III trial comparing nivolumab to chemotherapy in first-line PD-L1-positive metastatic NSCLC (CheckMate 026, NCT02041533) and a phase I multi-arm trial testing the safety and efficacy of nivolumab in NSCLC in combination with ipilimumab or standard chemotherapy or erlotinib or bevacizumab (CheckMate 012, NCT01454102).

Pembrolizumab (MK-3475), is a monoclonal anti-PD-1 humanized IgG4 antibody approved for the treatment of metastatic melanoma. Results from a phase I study in previously treated and untreated patients with advanced NSCLC showed that pembrolizumab was generally well-tolerated and provided robust antitumor activity. Overall response rate was 19.4% by RECIST 1.1 and the median duration of response was 12.5 months. Median PFS and OS were 3.7 and 12 months respectively. A proportion score of at least 50% of PD-L1 expression was associated with a higher response rate and longer PFS and OS than was a proportion score of less than 50%, which indicates that this is a subgroup of patients in whom the PD-L1 pathway can be successfully targeted. The toxicity profile was acceptable, with 9.5% of patients experiencing ≥ grade 3 adverse events. Pneumonitis of grade 3 or greater was observed in nine patients (1.8%), including one (0.2%) who died (40). Other trials are ongoing, including a phase II/III trial comparing 2 dose levels of pembrolizumab with docetaxel in PD-L1 positive NSCLC patients who have received ≥ one prior treatment regimen (NCT01905657) and other studies that compare pembrolizumab to platinum-based chemotherapy combinations as first-line treatment for PD-L1 positive NSCLC patients (NCT02142738 and NCT02220894).

Anti-PD-L1 drugs

Targeting PD-L1 with specific monoclonal antibodies has also shown to be a very promising approach similar to targeting PD-1. However, PD-L1 inhibition may result in different biological effects than those obtained from targeting PD-1: first, PD-L1 antibodies do not prevent PD-1 from interacting with PD-L2, although the effect of this interaction remains to be determined; second, PD-L1 blockade could also prevent the interaction of PD-L1 with the B7-1, thus suppressing an additional negative control on T cells (6,41).

BMS-936559 was the first anti-PD-L1 antibody demonstrating activity in a variety of advanced solid tumors. In a phase I trial, BMS-936559 treatment of NSCLC was associated with an objective response rate (ORR) of 10% (5 of 49 NSCLC patients evaluable: four patients with the non-squamous and one with the squamous subtype). Three of these patients had responses lasting at least 24 weeks.

Stabilization of disease at 6 months was observed in 12% of patients. In the overall patient population, grade 3 or 4 toxic effects related to treatment occurred in 9% of patients (42).

MEDI4736 is an engineered human IgG1 monoclonal antibody that blocks PD-L1 from binding to PD-1, thus allowing T cells to recognize and kill malignant cells. Interim results from the NSCLC cohort of an ongoing phase I, first-in-human study in patients with advanced solid tumors (NCT01693562), showed preliminary clinical activity of MEDI473 (43). At data cut-off, 155 NSCLC patients were treated with the drug in the dose escalation and expansion cohorts, of whom 143 received 10 mg/kg every 2 weeks. The majority of patients had received one prior systemic treatment and had non-squamous NSCLC. Early and durable activity was observed in both squamous and non-squamous NSCLC. Response rate by Response Evaluation Criteria in Solid Tumors (RECIST) was 13% and preliminary data indicated that PD-L1 expression may correlate with response and disease control rate. The drug was well tolerated, with no adverse events leading to discontinuation and low rates (4%) of grade 3/4 adverse events. No drug-related colitis and no grade 3/4 pulmonary toxicities were observed (43).

Ongoing trials are evaluating MEDI4736 in different settings of disease, including a phase III trial of the drug following concurrent chemoradiotherapy for unresectable stage III NSCLC (NCT02125461) and a phase III trial for pretreated, advanced NSCLC patients to test the combination of MEDI4736 with tremelimumab for PD-L1-negative patients and MEDI4736 as a single agent for PD-L1-positive patients *vs.* chemotherapy (NCT02352948).

Atezolizumab (MPDL3280A) is another engineered IgG anti-PD-L1 antibody, with modified Fc domain that prevents antibody-dependent cell-mediated cytotoxicity, that has shown activity in NSCLC. In a phase I trial, an ORR of 22% was observed in patients with previously treated metastatic NSCLC, with both squamous and non-squamous histology, including several patients exhibiting rapid tumor shrinkage (44). All responses were ongoing or improving at the time of analysis and the 24-week PFS was 46%. The safety profile was tolerable with no grade 3/4 pneumonities. In an updated analysis of the phase I trial, 85 patients with NSCLC were included in the safety analysis and 53 in the efficacy analysis. The ORR in the NSCLC cohort was 23% and all responses were maintained for the duration of treatment (median 48 weeks). Interestingly, the authors reported a higher ORR of 26% for patients who had smoked as compared with patients who had never smoked (ORR =10%). This could be explained by evidence demonstrating that, in comparison with non-smokers, smokers might bear tumors with a high mutation rate, significantly increasing their immunogenicity (45).

Biomarkers of response to PD-1/PD-L1 inhibitors

Identification of predictive biomarkers to select patients for immune therapies is currently being investigated; however it is difficult to establish the role of a single biomarker due to the complexity of the immune response which involves a great amount of interrelated molecules and immune cells, each of which dealing with a different crucial activity. Because of the role of PD-1/PD-L1 pathway in downregulating the activity of effector T cells within tumors and peripheral tissues, most biomarker investigations have focused on tumor microenvironment components. Tumor microenvironment is defined as being characterized by the crosstalk between different cell types, including tumor cells, inflammatory cells, T cells, B cells, NK cells, myeloid cell populations, tumor-associated fibroblasts (TAFs) and stromal cells. The most studied biomarker has been PD-L1 expression on tumor cells, due to its crucial role in immune response modulation by inhibiting the activity of tumor infiltrating cytotoxic T cells, thus creating a local immunosuppressive milieu.

Several studies have demonstrated that patients whose tumors express PD-L1, as detected by immunohistochemistry, have higher response rates to PD-1/PD-L1 blockade therapy than patients who do not express PD-L1 (35,40,43, 45,46). As commented above, activated tumor antigen-specific T cells produce interferons which induce PD-L1 expression on tumor cells. The presence of pre-existing PD-1 positive T cells with tumor antigen specificity which become inactivated upon binding with PD-L1 is crucial for responses to PD-1 blockade therapy (acquired immune resistance) (5). This mechanism differs from others in which tumor PD-L1 expression is not correlated to the presence of effector cells and to PD-1 expression on these cells. Indeed, tumor cells can activate PD-L1 expression independently of inflammatory signals via multiple oncogenic signaling pathways, including PI3K/AKT, ALK/STAT3, MEK/ERK/STAT1 (defined as innate immune resistance) (47-49).

However, in many of these trials, albeit at a lower rate, responses have also been observed in patients with PD-L1-negative tumors, suggesting that other factors could explain immunotherapy activity in this subgroup of patients. In a seminal study exploring the predictive

value of multiple immune biomarkers in pretreatment samples from nivolumab-treated patients with different advanced solid tumors, PD-L1 expression on tumor cells emerged as the strongest predictive factor associated with response to the antibody (46). The presence of TILs was not demonstrated to be predictive of anti-PD-1 response, in contrast to others demonstrating a correlation of the numbers of TILs with ipilimumab activity in melanoma (13). In a recent study, Herbst *et al.* observed that the association of PD-L1 expression by infiltrating immune cells with objective response to the anti-PD-L1 monoclonal antibody MPDL3280A was stronger than that with tumor PD-L1 expression, suggesting that a pre-existing T-cell activity suppression could be crucial in mediating response to the anti-PD-L1 monoclonal antibody (50). However, results in terms of PD-L1 expression predictivity to immunotherapies are difficult to interpret, because of the lack of a standardized immunohistochemistry assay, different cut-off levels to determine PD-L1 positivity, variability in the timing of biopsy collection, in sample processing and preservation across all the reported studies. In addition, tumors are heterogeneous and the sample used for the assay may not be representative of the whole tumor. Other potential predictive biomarkers of response to immunotherapies have been identified, including the presence of CD8+ T-cell infiltration and the expression of PD-L1 and PD-1 in immune cells at the invasive tumor margin that correlated with response to pembrolizumab in melanoma (51). Recent evidence suggests that response to immunotherapies may rely on a tumor specific genomic landscape. Indeed, every tumor contains a variable number of somatic mutations and some tumors, including melanomas and lung cancers, are characterized by very high numbers of somatic mutations associated with environmental exposure to ultraviolet light and smoking. Somatic mutations can generate neoantigens within tumors that can trigger specific immune responses. A higher mutational load as assessed by whole-exome sequencing in NSCLC samples from patients was strongly associated with response to pembrolizumab. Efficacy was also correlated with a molecular smoking marker, specific DNA repair pathway mutations, and a higher burden of candidate neoantigens (52). Epithelial-mesenchymal transition (EMT) has been correlated with cancer progression, metastasization and drug resistance. In a recent work, it was demonstrated that EMT is correlated with CD8+ TIL immunosuppression and metastasis through a mechanism involving ZEB1 and miR-200 loss that controls PD-L1 expression in lung cancer cells (53).

TGF-β signalling pathway

The TGF-β superfamily are cytokines that bind to a heterodimeric receptor complex consisting of type I (TβRI, activin-line receptor kinase family) and type II (TβRII) transmembrane serine/threonine kinase receptors. TGF-βs are secreted by tumor cells, immune cells and other nonhematopoietic cells; three isoforms of TGF-β have been identified of which TGF-β1 is predominantly expressed in the immune system (54). The TGF-β ligands and receptors are widely expressed in different tissues and tumors, playing an important role in the maintenance of immune homeostasis and the coordination of responses to injury and stress. Unlike most cytokines, TGF-β is synthesized as an inactive precursor, which forms a homodimer that interacts with its latency-associated peptide (LAP) and a latent TGF-β-binding protein (LTBP), forming a larger complex called the large latent complex (LLC). The TGF-β activation process involves the release of the LLC from the extracellular matrix (ECM), followed by further proteolysis of LAP to release active TGFβ to its receptors. Matrix metalloproteinase 2 (MMP2), MMP9 and thrombospondin 1 (THBS1) are known to cleave latent TGF-β. Alternatively, interaction with integrins or pH changes in the local environment are known to activate latent TGF-β and free active TGF-β (55). Binding of TGF-β with the heterodimeric receptor complex activates intracellular signaling involved in the control of crucial mechanisms, including proliferation, differentiation, migration, invasion, EMT, ECM remodelling and immune-suppression (56). Intracellular signal transduction is mediated by phosphorylation of specific transcription factors known as Smads (57). Phosphorylated Smad2 and Smad3 combine with Smad4 to modulate gene transcription by entering the nucleus, binding to the target genes promoters and recruiting histone acetyl transferase or histone deacetylase. Smad7 is a negative regulator of the Smad signalling pathway, that binds to ubiquitin specific peptidase 15 (USP 15) as a complex with SMAD specific E3 ubiquitin protein ligase (SMURF2), leading to enhanced TGF-β signalling. Other Smad-independent signalling molecules and pathways can be regulated by TGF-β, including Ras-Mek-Erk, Rho GTPase, PI3K/AKT, TGF-β-activated kinase 1 (TAK1), p38, JUN N-terminal kinase (JNK) and nuclear factor-κB. (NF-κB) (58). TGF-β is a pleiotropic

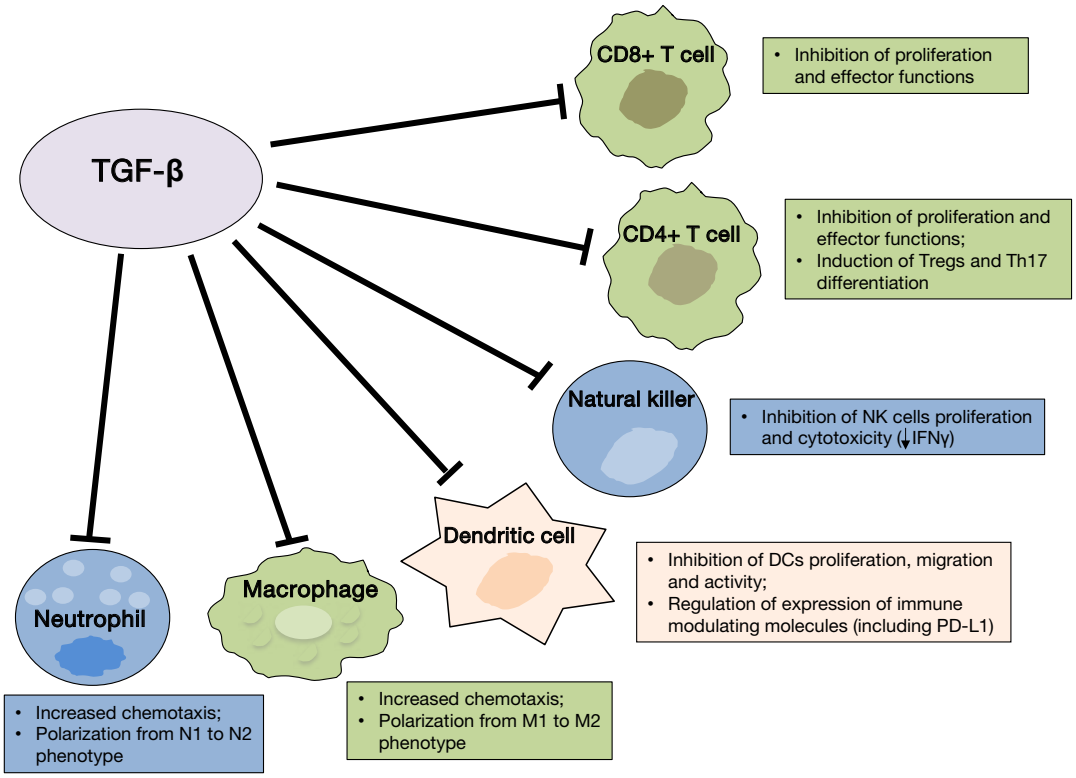

Figure 1 Immune cellular targets of TGF-β. TGF-β, transforming growth factor-β; NK, natural killer; IFN, interferon; DCs, dendritic cells; PD-L1, programmed cell death ligand-1.

cytokine that exerts its function on several cells types; therefore, dysfunction of TGF-β signalling could play a role in multiple biological processes and pathologies, including carcinogenesis, fibrosis, wound healing and immune responses (54,55,59-61).

The role of TGF-β in immune response

TGF-β has a pivotal role within the immune system; it is mainly involved in suppressing the immune response in the periphery, thus maintaining immune self-tolerance and preventing autoimmune disease. Therefore it has been correlated with host immunosurveillance inhibition. As a pleiotropic cytokine, TGF-β maintains immune homeostasis through regulation on essentially every cell type of the innate and adaptive immune system (*Figure 1*). Specifically, TGF-β suppresses the proliferation, differentiation and effector functions of multiple immune cell types, especially T lymphocytes, and induces the generation of immunosuppressive cells or phenotypes (55,62). Experiments in transgenic mice have demonstrated the critical role for TGF-β in regulating suppression of conventional CD4+ and CD8+ T cells. A direct correlation between the frequency of cytotoxic T lymphocytes (CTLs) in TILs, mainly activated cytotoxic CD8+, and the OS has been demonstrated in patients with different types of cancer (9-12,63). TGF-β signalling dampens tumor-specific CTLs function and frequency in tumors and blocking TGF-β signalling on CD4+ and CD8+ T cells is associated with a strong antitumor response due to enhanced proliferation and activity of tumor-specific CTLs (62,64).

TGF-β has been demonstrated to exert its activity by regulating T cell proliferation, differentiation and survival through multiple mechanisms. TGF-β has been shown to suppress T-cell proliferation through Smad3 transcription factor-dependent blockade of the production of IL-2, which is a known cytokine able to activate T cells, NK, and other types of cells of the immune system (54,65). However, other IL-2-independent mechanisms involved in T-cell proliferation inhibition have been described, including Smad3 binding to myc-promoter and decreased expression of other cell-cycle promoting factors and up-regulation

of cyclin-dependent kinase inhibitors, including p15 and p21 (54,66). TGF-β markedly suppresses the "cytotoxic program" of CTLs through transcriptional repression of genes encoding key proteins involved in the effector functions, including perforin, granzymes, IFN-γ and other cytotoxins (62,67). Recently, one of the mechanisms involved in T cell unresponsiveness driven by TGF-β has been described in cancer cell lines and mice models (68). In this study, upregulation of the forkhead box protein 1 (Foxp1) was demonstrated in human and mouse tumor-infiltrating effector CD8+ T cells. Foxp1 inhibited T-cell proliferation and impaired T-cell effector functions by decreasing cytolytic Granzyme-B and IFN-γ production in response to antigens. These effects were mediated by interaction of Foxp1 with Smad2 and Smad3, induced in response to microenvironmental TGF-β. These results suggest Foxp1 is required for the suppressive activity of TGF-β on CD8+ T cells, including transcriptional repression of c-myc and c-jun (68).

TGF-β also contributes to immunosuppression because of its significant impact on CD4+ T-cell differentiation and functions. Indeed, TGF-β, as well as other suppressing cytokines present at high concentrations in tumor microenvironment, have a crucial role in inducing Treg cells generation (55,62). Tregs are diverse populations of lymphocytes that physiologically maintain peripheral tolerance and immune homeostasis. These cells are found at higher frequencies in peripheral blood, lymph nodes and tumor sites of cancer patients and can suppress antitumor immune response (54,62,69). Tregs are divided mainly in two distinct subsets: natural Treg (nTreg) cells and adaptive or induced Treg (iTreg). nTreg are thymus-derived T cells that arise during early stages of human fetal development and maintain self-tolerance in an antigen-independent manner. iTreg cells, which include a great variety of cells stimulated by different cytokines, are induced in the periphery (iTreg) from naïve T cells in response to self- or tumor-antigens stimulation (70,71). Tregs are CD4+ cells characterized by the expression of CD25, the alpha chain of IL-2 receptor. The expression of trascription factor Foxp3 has been demonstrated to be necessary for the development and proper function of these cells (71,72). nTreg also express other markers, including adhesion molecules, CTLA-4, PD-1, chemokine receptors (CCR7, CXCR4), CD28 and GITR-related protein (5,71,73). These cells are able to suppress proliferation and cytokine secretion of conventional CD4+ effector T cells and they can also suppress other immune cell types, including CD8+ T cells, NK cells, monocytes/macrophages, B cells (71,74).

Different studies *in vitro* have demonstrated that TGF-β induces up-regulation of Foxp3 expression in T cells stimulated via TCR, thus generating cells with suppressive properties (iTreg). IL-2 is necessary for the conversion of naïve T cells to Foxp3+ T cells (75,76). NF of activated T cells (NFAT) and Smad3 are crucial for Foxp3 induction in iTregs, however, Foxp3 expression can also be positively regulated by relief from GATA-3 mediated transcriptional inhibition of its promoter by competition with the TGF-β-induced Id3 (77,78). TGF-β could be involved in generating Tregs *in vivo* and may assist these cells in suppressing CTL effector function in the tumor microenvironment, as demonstrated in mouse models (54,62). High levels of Tregs within tumors have been correlated with poor prognosis in several tumor types, including lung cancer (79-81), but the role of TGF-β in Tregs generation in cancer patients remains to be determined. Since several tumors can produce TGF-β, the induction of Treg cells by this cytokine might be a mechanism by which tumors escape the antitumor immune response, thus suggesting new potential anti-tumor immunotherapeutic strategies. However, by using microarray techniques, it was demonstrated that the signature of genes expressed by TGF-β-induced-Foxp3+ T (iTreg) cells differed from that of nTreg, suggesting the need for further studies before assessing their potential role for therapeutic approaches to human diseases (75).

In addition, TGF-β inhibits Th1 and Th2 cell differentiation which are crucial in increasing the CTL-mediated antitumor response and, together with IL-6, promotes Th17 cell differentiation (55,82,83). It is presumed that IL-6 inhibits Tregs development with stimulation of Th17. TGF-β, coordinating with IL-21, induces CD4+, CD25+ Tregs that counterbalance the effect of IL-6. Th17 is a newly defined Th-cell population that expresses IL-17 and regulates leukocyte recruitment and activation. However, the functions of Th17 cell types in tumor biology remains controversial and also the role of TGF-β in Th17 differentiation has been debated in more recent studies (55,84). TGF-β has been demonstrated to also affect B-cell, by suppressing their proliferation and modulating their activation and secretion of Immunoglobulins (62).

DCs are the most powerful antigen-presenting cells (APCs) and they are able to initiate antigen-specific immune responses by presenting antigens to T cells and by activating B and NK cells. DCs are at the center of the immune system owing to their ability to control both tolerance and immunity (85). Upon stimulation, DCs up-

regulate MHC II, costimulatory receptors and cytokines, including IL-12, and interact with T-cells in order to induce their activation and differentiation. TGF-β affects DC biology in several ways (62). TGF-β can reduce DCs motility, thereby interfering with the migration and antigen transportation to draining lymph nodes for presentation to adaptive immune cells and it can also directly induce DC apoptosis. DCs secrete TGF-β and respond to TGF-β produced by tumor cells, either in autocrine or paracrine manner, by down-regulating expression of MHC II and co-stimulatory molecules and cytokines, including CD40, CD80 and CD86, tumor-necrosis factor, IFNα, IL-12 and CC-chemokine ligand 5 (CCL5) (62,86). These tolerogenic DCs are able to promote the formation of Tregs and this capacity is increased by TGF-β (62,87-89). These effects may result in inhibition of T-cell activation and contribute to the generation of a local immune suppressive milieu and tumor escape from immune system.

In *in vitro* experiments exploring the role of TGF-β in lung tumor microenvironment, TGF-β also upregulated the expression of immune modulation molecules, including PD-L1 and GITR-related protein ligand, on DCs (90). In a recent work, TGF-β was shown to upregulate PD-L1 expression on DCs via activation of STAT3 phosphorylation and signaling activation (91). Several studies have linked PD-L1 expression on DCs with the induction of immune tolerance and tumor evasion from immune response. The binding of PD-L1 with PD-1 expressed on T cells is responsible for T-cell immune activity suppression. PD-L1 expression has been demonstrated to be linked to the development and activation of Tregs. In this study, TGF-β-DCs were able to induce CD4+, CD25+, Foxp3+ Treg generation and apoptosis of T cells, thus diminishing the ability of DCs to activate a tumor-antigenic specific CTL response. A positive correlation between Treg percentage and PD-L1 expression was found (91).

With respect to innate immunity system cells, TGF-β inhibits NK-cell proliferation and function. TGF-β directly suppresses IFNγ production by NK cells through transcriptional effects of SMAD3 on the IFNγ promoter (92). Furthermore, TGF-β inhibits NKp30 and NKG2D receptor expression, resulting in decreased NK-cell cytolytic activity (93,94).

TGF-β also has a role in regulating myeloid lineage cells within tumors. Solid tumors are composed of approximately 50% macrophages and high levels of tumor-associated macrophages (TAMs) are correlated with poor cancer prognosis. TAMs are a heterogeneous population and are typically divided in different phenotypes with different activities: M1 are activated by IFN-γ and lipopoly saccharide (LPS) and are efficient at presenting antigens and have immunostimulating activity by secretion of active cytokines, including IL-12; in contrast, M2 include a variety of macrophages exerting suppressive function, that are mainly involved in wound healing and promote angiogenesis and tumor progression, similar to myeloid-derived suppressor cells; M2 are mainly induced by IL-10, IL-4 and IL-13 (95-97). TAMs acquire their phenotype by expressing high levels of TGF-β, IL-10, CXC-chemokine ligand 10 (CXCL10) and CXCL9 and other IFN-γ-responsive genes. In skin cancer, TGF-β recruits TAMs and this contributes to immune escape and tumor progression (98). TGF-β promotes M1 to M2 phenotype differentiation and this can be associated to down-regulation of NF-κB expression and activation (62). TGF-β also affects neutrophils migration and cytotoxicity (99,100). Neutrophils have important functions during inflammation and, although their role in tumor progression needs to be determined, those infiltrating tumor associated neutrophils (TANs) are polarized into two different phenotypes (N1 and N2), similar to TAMs. N2 cells promote tumor angiogenesis and metastasis and produce reactive oxygen species (ROS) and pro-inflammatory cytokines that inhibit the antitumor immune response by leading to oxidative damage and inhibition of T-cell function. TGF-β has been demonstrated to influence polarization of neutrophils within tumor microenvironment (62,101).

Gr-1+, CD11b+ immature myeloid cells are also called myeloid derived suppressor cells (MDSCs) and play an important suppressive effect through inhibition of T-cell activation but also NK, DCs and B cells, through the production of arginase and ROS. Tumors utilize numerous pathways to inhibit immune responses, including the elaboration of immune-suppressive mediators such as prostaglandin E2 (PGE2), TGF-β, IL-10, vascular endothelial growth factor (VEGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6 and stem cell factor (SCF), which recruit and/or activate MDSCs. Solid tumors contain MDSCs that maintain an immune-suppressive network in the tumor microenvironment and compromise the efficacy of cancer immunotherapy (102,103). MDSCs could also affect tumor progression; in fact, they produce high levels of MMPs and TGF-β and contribute to tumor growth, neovascularization and metastasization (104,105).

The role of TGF-β in tumor progression and EMT

High levels of TGF-β are produced by many hematologic and solid tumors, including breast, colon, liver and lung and TGF-β overexpression has been correlated with metastasis, poor prognosis and resistance to therapy. In addition, mutations of TGF-βRs as well as Smad proteins have been found in several cancer types (54,59,106). During tumorigenesis, TGF-β acts both as a tumor suppressor in earlier stages and as a tumor promoter during later stages (54,107-109); the mechanisms underlying this dual role remain to be elucidated. In early stages, TGF-β can suppress tumorigenesis by inhibiting cell cycle progression and stimulating apoptosis. In contrast, it has been reported that later stages of cancer progression are characterized by increased expression or activation of the TGF-β pathway by tumor cells in which it contributes to modulate cancer-inducing processes. One of the hallmarks of cancer is that the vast majority of cases exhibits insensitivity to TGF-β-mediated growth inhibition. TGF-β is involved in EMT induction, angiogenesis and metastasization. As mentioned above, EMT enhances cellular migration and invasive properties, as cell migration requires loss of cell-cell contacts and acquisition of fibroblastic characteristics. EMT can also be associated with the acquisition of cancer "stem cell-like" properties and, therefore, more aggressive tumor cells and worse response to treatments, including radiotherapy and chemotherapy (110,111). Recently, NSCLC samples with EMT features, as determined by a specific gene expression signature, were found to be strongly associated with upregulation of multiple immune-activating and immune-modulatory molecules, including IL-6, IL-10, CTLA-4, IDO, compared to epithelial NSCLC samples. In addition, EMT was associated with higher expression of PD-L1 on tumor cells, suggesting that immunotherapies targeting immune checkpoints might be beneficial for these subgroups of NSCLC patients with mesenchymal characteristics (112).

The anti-tumor immunity suppression by TGF-β may contribute importantly to cancer progression and resistance to existing therapies. In experiments in melanoma cell lines and murine model, a correlation between hypoxia-induced upregulation of the stemness-associated transcription factor Nanog, TGF-β and immunosuppression has been demonstrated (113). Hypoxia is commonly observed in tumor microenvironment and it is responsible of acquisition of stem-cell like properties by tumor cells and promotion of tumor tolerance through regulation of multiple immune cells. Nanog was involved in the control of tumor growth following treatment with specific therapeutic vaccination (114). The same group demonstrated that hypoxia-induced Nanog correlated with acquisition of stem cell-like properties in tumor cells. Furthermore, under hypoxic conditions, it enhanced TGF-β expression by binding with its promoter and, through TGF-β, promoted the differentiation of CD4+ T naïve cells into Tregs and the macrophages immunosuppressive phenotype. Nanog targeting significantly reduced Tregs and macrophages and increased CD8+ T effector cells infiltrating in tumor bed, thus suggesting new potential strategies to enhance immune therapies.

Conclusions

Immune checkpoint inhibition is a promising strategy in lung cancer, inducing durable responses with manageable toxicities. However, the modest ORR suggests the need to further improve their therapeutic efficacy. To this aim, it is of paramount importance to identify predictive biomarkers to select target patients that may most benefit from checkpoint blockade. Immune response is regulated by many molecules and immune cells within the tumor microenvironment, which can also have a role in modulating response to anti-PD-1 and anti-PD-L1 monoclonal antibodies. PD-L1 expression in tumor cells has emerged as the most promising candidate for predicting response to these drugs, however, the lack of PD-L1 expression can not be relied upon to exclude patients from these highly effective immunotherapeutic approaches. The role of other components of tumor microenvironment in modulating the response to anti-PD-1 and anti-PD-L1 antibodies is being deeply investigated. TGF-β is a tolerogenic cytokine that is frequently overexpressed in aggressive cancers and plays a crucial role in the maintenance of immune homeostasis and in tumorigenesis. Its pivotal function is to suppress the immune response in periphery through its multiple effects on several cell types. TGF-β has suppressive effects on Th1 and cytotoxic T-cells, while promoting the generation and activity of Treg cells. In innate immunity, TGF-βs increase the suppressive activity of monocytes/macrophages by promoting polarization towards an M2 phenotype and decrease the cytotoxicity of NK cells. In addition, TGF-β affects antigen presentation by DCs by modulating the expression of MHC II and also induces upregulation of immune modulating molecules, including PD-L1, with the induction of immune tolerance and tumor evasion

from immune response. Furthermore, TGF-β has been correlated with EMT phenotype that can play a crucial role in cancer progression and resistance to treatments. Recently, lung cancers with EMT phenotype have been demonstrated to have higher expression of PD-L1 and other markers of inflammatory response. A close relation exists between the immunologic system, hypoxia, acquisition of stemness properties, and EMT induced by TGF-β, thus suggesting new opportunities for enhancing immune therapies. Although some evidence suggests a potential role for TGF-β in modulating response to specific anti-PD-1/PD-L1 monoclonal antibodies, due to the pleiotropic activity of TGF-β, its role in immunoresistance is highly complex and context-dependent and still needs to be explored. Indeed, preclinical studies have shown that TGF-β antagonists can significantly suppress tumorigenesis and/or metastasis by effective antitumor immunity reactivation mechanisms. For the same reasons, TGF-β antagonism can be able to improve the efficacy of immunomodulatory chemotherapy and of immunotherapeutic agents. Understanding the intricate signalling pathways and multiple biological processes controlled by TGF-β could provide new insights about the role of microenvironment during immune response. However, the role of TGF-β inhibitors, also in association with vaccines or anti-PD-1/PD-L1 or CTLA-4 specific antibodies and the optimal timing of administration of these immunotherapies in the clinical setting still need to be determined.

Beyond TGF-β, deeper understanding of the immune features of lung cancers will contribute to the identification of more reliable predictive markers for assessing response to checkpoint inhibitor therapy.

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None.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Shedding light on the molecular determinants of response to anti-PD-1 therapy

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Provenance: This is a Guest Editorial commissioned by the Section Editor Hongbing Liu (Department of Respiratory Medicine, Jinling Hospital, Nanjing University School of Medicine, Nanjing, China).

Abstract: Immune checkpoint inhibition targeting the programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1) axis has emerged as a very promising therapeutic avenue in the treatment of patients with advanced stage non-small cell lung cancer but only a subset of patients derives clinical benefit from PD-1/PD-L1 inhibitors. Studies to date have reported patients with PDL-1 expressing tumors have a better outcome than those with PDL-1 negative tumors but assays used to identify PD-L1 positive patients has been challenging. Through whole exome sequencing of tumors, investigators have recently described mutational burden in non-small cell lung cancer (NSCLC) was associated with response to pembrolizumab. In two independent patient cohorts, it was reported a high somatic nonsynonymous mutation burden was associated with greater durable clinical benefit, higher objective response rates (ORRs) and a longer progression free survival. In addition clinical efficacy was associated with a molecular smoking signature, certain DNA repair mutations and the burden of neoantigens.

Keywords: Programmed death-1 (PD-1); programmed death-ligand 1 (PD-L1); immunotherapy; whole exome sequencing; non-small cell lung cancer (NSCLC)

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Progress in the understanding of the role of the immune system in tumor immunosurveillance has led to the recognition that tumors can evade immune destruction via the dysregulation of co-inhibitory or checkpoint signals. Lung tumorigenesis is not only dependent on genetic aberrants within cancer cells but also on interactions with the immune system (1). In the normal physiological state, programmed death-1 (PD-1), an immune checkpoint or co-inhibitory molecule found on activated T cells, acts to prevent autoimmunity (2). The binding of PD-1 with one of its ligands, programmed death-ligand 1 (PD-L1) (or CD274, B7-H1) or PD-L2 (CD 273, B7-DC), leads to the down-regulation of cytotoxic T cell function (2). However tumors can co-opt the PD-1/PD-L1 pathway to evade T-cell–induced antitumor response (2,3). The inhibition of the PD-1/PD-L1 pathway with immune checkpoint inhibitors can interrupt the engagement of PD-1 with its ligands and block inhibitory signals in T cells, resulting in tumor recognition by cytotoxic T cells.

The development of immune checkpoint inhibitors such as nivolumab, pembrolizumab (PD-1 inhibitors) and MPDL3280A (PD-L1 inhibitors) represent an important breakthrough in the treatment of cancer. Early phase studies of PD-1/PD-L1 inhibitors have reported impressive clinical activity and durable responses in patients with refractory tumors including melanoma, renal cell cancer, Hodgkin's lymphoma, bladder cancer, and non-small cell lung cancer (NSCLC) (4-10) and recent phase III studies have reported pembrolizumab and nivolumab conferred OS benefit in melanoma versus ipilimumab (11) and dacarbazine (12)

Table 1 ORR of PD-1/PD-L1 inhibitors in patients with advanced stage NSCLC according to smoking status and histologic subtype

Immune checkpoint inhibitor	ORR (95% CI)				Reference
	Smoking status		Histologic subtype		
	Never	Former/current	Squamous	Non squamous	
Nivolumab	0 [0-25]	27 [17-38]	16.7 [7.9-29.3]	17.6 [9.7-28.2]	(15)
Pembrolizumab	10.3 [5.6-17]	22.5 [18.3-27.1]	23.5 [15-34]	18.7 [15-22.9]	(10)
MPDL3280A	10	42	27 [8-61]	21 [11-36]	(8)

ORR, objective response rate; PD-1, programmed death-1; PD-L1, programmed death-ligand 1; NSCLC, non-small cell lung cancer; CI, confidence interval.

Table 2 ORR of PD-1/PD-L1 inhibitors in patients with NSCLC according to PD-L1 IHC assay

Drug	Nivolumab			Pembrolizumab				MPDL3280A			MEDI4736
Pharmaceutical	BMS			Merck				Roche/Genentech			Astra-Zeneca/ MedImmune
Ab clone (source)	28-8 (Dako)			22C3 (Dako)				SP142 (Ventana)			SP263 (Ventana)
Tissue	Archived			Recent				Archived/recent			Archived/recent
Cell scored	Tumor cell membrane			Tumor cell or stroma				Infiltrating immune cells			Tumor cell membrane
Study (reference)	CheckMate (9,15,21)			KEYNOTE-001 (10)				NCT01375842 (8)			NCT01693562 (22)
	012		063								
Treatment line	1st	≥2nd line	≥2nd line	1st		≥2nd line		≥2nd line			≥2nd line
Cutoff	5%	1%	5%	5%	1-49	≥50	1-49	≥50	1-<5	≥5-<10	≥10	NR
ORR (%) in PD-L1 +ve	50	13	15	24	19.2	50	15.6	43.9	15	14	83	39
ORR (%) in PD-L1 –ve	0	17	14	14	17		9.1		20			5

ORR, objective response rate; PD-1, programmed death-1; PD-L1, programmed death-ligand 1; NSCLC, non-small cell lung cancer; IHC, immunohistochemistry.

respectively and improvement in OS in patients with advanced stage NSCLC treated with second line nivolumab versus docetaxel (13,14).

As clinical efficacy is seen only in a subset of patients with advanced stage NSCLC treated with PD-1/PD-L1 inhibitors, biomarkers are needed to improve patient selection for immune checkpoint inhibition. Whilst histologic subtype was not associated with response, current or former smoking status has been reported to be associated with an increased response to treatment in several studies (*Table 1*). A possible explanation for this finding is smoking-associated lung cancers have a higher mutational load, resulting in the creation of more tumor neoantigens and increased immunogenicity (16,17).

Since the PD-1/PD-L1 pathway is implicated in immune escape in NSCLC, tumor PD-L1 expression with immunohistochemistry (IHC) has been used as a predictive biomarker. In one study, no association between tumor PD-L1 expression and response to nivolumab was found (15) whereas in KEYNOTE 001, tumor PD-L1 expression was associated with response to pembrolizumab (10). A possible confounder was PD-L1 expression was determined using archival tumor samples in the nivolumab study whereas a fresh tumor biopsy was obtained in the latter study.

The development of anti-PD-L1 IHC companion diagnostics by several pharmaceutical companies has created some challenges as these assays are using different IHC antibody clones, different staining protocols and platforms, different scoring systems, and different cutoffs defining positivity. Other issues influencing PD-L1 assessment include tumor heterogeneity, the dynamic nature of PD-L1 expression (18-20) and differences in the assessment of PD-L1 in the tumor microenvironment (tumor cell, stroma, or both) (*Table 2*). Regarding the latter factor, PD-L1 expression in the tumor microenvironment may be an important determinant of response. Herbst and colleagues

found PD-L1 expression in tumor infiltrating immune cells, instead of tumor cells, was associated with response in patients with advanced stage NSCLC treated with MPDL3280A (8) whereas immune cells at the invasive margin expressing PD-1 and PD-L1 was associated with response in patients with advanced stage melanoma treated with pembrolizumab (19). Furthermore, whilst high PD-L1 expression is associated with a higher response rate, responses of 5-20% are also seen in PD-L1 negative patients, adding further complexity to using PD-L1 as a biomarker (*Table 2*).

As immune checkpoint inhibitors are active in bladder cancer (6), NSCLC (8,10,15), and melanoma (11,12), tumors typically associated with high somatic mutation rates (16), it has been hypothesized that the mutational landscape is a determinant of response to PD-1/PD-L1 inhibitors. Based on this premise, investigators have recently reported mutational burden in NSCLC was associated with response to PD-1 inhibition. In this seminal Science paper, the subject of this editorial, Rizvi and colleagues performed whole-exome sequencing of tumors from a phase I study of patients with advanced stage NSCLC treated with pembrolizumab (23). In two independent patient cohorts they found a high somatic nonsynonymous mutation burden was associated with greater durable clinical benefit (defined as a partial or stable response of at least 6 months), higher objective response rates and a longer progression free survival. In addition, clinical efficacy was associated with a molecular smoking signature, certain DNA repair mutations and the burden of neoantigens. Interestingly the molecular smoking signature, rather than self-reported smoking status, correlated with efficacy.

Furthermore, a high nonsynonymous mutational burden correlated with a greater number of putative neoantigens with high binding affinity to patient-specific HLA alleles, and patients who had a durable clinical benefit had a higher neoantigen burden than those who did not. These findings are significant as they support the hypothesis that the recognition of neoantigens, formed as a result of somatic mutations, is important for anti PD-1 activity and provides further proof of principle that tumor genomics can dictate responses to immunotherapy. The same investigators have recently reported mutational load and a neoantigen landscape that is specifically present in melanomas was associated with response to CTLA-4 inhibition (17). Another notable finding is T-cell response against a mutation-associated neoantigen was detected in peripheral blood lymphocytes from one patient who responded to pembrolizumab. This important finding creates an opportunity to develop blood-based assays to monitor response to PD-1 inhibition.

What is unknown from this study is the correlation between mutational load and PD-L1 density and more studies are needed to determine the relationship between mutational burden and PD-L1 expression. Further studies are also required to confirm these findings with other PD-1/PD-L1 inhibitors and in other malignancies. Research in identifying mechanisms of resistance to immune checkpoint inhibitors will also aid in the development of rational therapeutic strategies and ultimately improve patient outcomes. As PD-L1 IHC assays may be introduced into clinical use in the near future, the International Association for the Study of Lung Cancer is planning an international characterization study of PD-L1 companion diagnostics to gain a better understanding of the assays, their performance on different sample types (large specimens, small biopsies, cytology) and different on platforms.

In summary, through a better understanding of the molecular determinants of response to immunotherapy, Rizvi *et al*. has provided insight on how tumor mutational load in NSCLC influence the efficacy of anti-PD-1 therapy and T-cell responses to neoantigens created by somatic mutations might underpin pembrolizumab activity in NSCLC. The results also underscore the importance of incorporating biomarkers in studies to characterize molecular mechanisms of sensitivity.

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Footnote

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Predictive factors of activity of anti-programmed death-1/ programmed death ligand-1 drugs: immunohistochemistry analysis

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Abstract: Anti-programmed death-1 (anti-PD-1)/programmed death ligand-1 (PD-L1) therapeutic antibodies targeting regulatory pathways in T cells have recently shown to promising clinical effectiveness in several solid tumors by enhancing antitumor immune response. Immune checkpoint therapy has propelled therapeutic efforts opening a new field in cancer treatment. However, durable clinical response has been educed only in a fraction of patients, underlining the need to predictively select those patients most likely to respond, e.g., by detecting predictive biomarkers. Immunohistochemistry (IHC) detection of PD-L1 in tumor cells has been used in various trials of anti-PD-1/PD-L1 agents to try to select those patients most likely to respond. However, since there are different techniques and scoring systems, results have not been conclusive. Thus efforts are needed to develop standardized IHC assays as well as to explore additional biomarkers to evaluate and predict immune responses elicited by anti-PD-1/PD-L1 therapies.

Keywords: PD-1; programmed death ligand-1 (PD-L1); immunohistochemistry (IHC); biomarker; immunotherapy

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Introduction

Lung cancer currently remains the leading cause to cancer-related deaths globally; each year 1.8 million people are diagnosed with lung cancer (1). Lung cancer has traditionally been classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), with latter accounting for about 85% of all cases. Despite development of clinical diagnostic techniques, approximately 75% of all the NSCLC cases are diagnosed at an advanced or metastatic stage, thus leading to poor prognosis. With limited successful treatment options, surgery remains the standard treatment in the early stages of the NSCLC; for aggressive NSCLC, first-line of treatment is chemotherapy.

In the past decade, there have been several studies highlighting the role of EGFR and its mutations in lung tumorigenesis and response to EGFR small-molecule inhibitors (2). Even though identification of treatable mutations and development of new molecular targeted agents, the number of patients with these aberrations with access to these medications remain less than 20% (3). Thus, the prognosis of NSCLC remains grim, with a 5-year overall survival of high-stage patients approximately of 1% (4). Obviously, there is great need for a novel therapeutic approach that could improve the outcome for these NSCLC patients.

During the past few decades, there have been great advances in the understanding of the interaction between

immune system and tumor progression. As such, lack of immunological control has been recognized as one of the tenets of cancer. Currently, there is increasing evidence pointing towards the formation of a tumor immune microenvironment that fosters tumorigenesis. Tumor cells disrupt host immune checkpoints thus escaping immune surveillance and avoiding their elimination from the host immune system. Transformation of normal to malignant cell involves several genetic and epigenetic changes, which can produce neoantigens that could be recognized by host immune system, thus triggering the body's immune response. Under normal physiological conditions, T-cells would recognize cancer cells as abnormal and populate cytotoxic T-lymphocytes (CTLs) at the site thus infiltrating and killing cancer cells in the process. However, cancer cells utilize several pathways to up-regulate negative signals blocking T-cell activation and induction of cell death, thus promoting tumor growth and eventual metastases. Increasing number of studies and clinical trials show that an immunotherapeutic approach to utilizing antibodies against immune checkpoint modulators can release inhibitory signals and facilitate antitumor activity of the immune system.

In the current review, we would include the PD-1/programmed death ligand-1 (PD-L1) signaling in lung cancer and current efforts to employ immunohistochemical analysis to predict clinical response to anti-programmed death-1 (anti-PD-1) and anti-PD-L1 medications.

Tumor immunogenicity

Early trials with various vaccines and immunostimulatory agents such as interleukin-2 that induces CTL proliferation failed to achieve satisfactory therapeutic outcome (5). Only a limited subset of NSCLC patients benefited with moderate survival benefits. However, there were immune-related side effects (6) leading to the belief that lung cancer is insensitive to immunotherapy.

In contrast, recent clinical data indicate that lung cancer is a suitable candidate for immunotherapy; patients with increased tumor-infiltrating lymphocytes (TILs), CTLs, T-helper cells, dendritic cells (DCs) and natural killer (NK) cells, have been shown to have a better prognosis (7-9). Lung tumors have also been shown to have high population of immunosuppressive cells like T-regulatory cells (T-regs) and myeloid-derived suppressor cells (MDSCs) thus disrupting killer cell activity (10). In addition, lung cancer cells secrete cytokines, interleukins, prostaglandins and growth factors attenuating T-cell response (11-14). There is also decrease in the levels of MHC class I expression in lung tumors facilitating the escape from routine antigen processing and evading immune surveillance (15). There are new and exciting data showing an improved rate of clinical response to antibodies targeting immune checkpoints (16). One of the goals of such immune checkpoint therapy is to remove the inhibitory pathways that block antitumor T cell response. Thus, the mechanism differs from traditional therapies targeting tumor or tumor vasculature, and earlier immunostimulatory.

Currently, research work in the field of immune checkpoint therapy is mainly focused on PD-1 and its ligand PD-L1 (B7H1). PD-1/PD-L1 axis regulates T cell activation in the later stages of tumor development. Antibodies targeting the PD-1/PD-L1 pathway have shown clinical responses in various cancers including NSCLC (17,18).

PD-1 and PD-L1

PD-1 was initially discovered while characterizing the role of *de novo* RNA synthesis in the apoptotic cell death of murine thymocytes (19), and earlier studies showed that it is a type 1 trans-membrane protein belonging to extended CD28/CTLA-4 immunoglobulin family and encoded by the PDCD1 gene (20). PD-1 is one the most important inhibitory co-receptors expressed on activated T cells. The PD-1 molecule comprises of an extracellular IgV domain, a hydrophobic transmembrane region, and an intracellular domain containing potential phosphorylation sites that are located in the immune tyrosine-based inhibitory motif (ITIM) and immune receptor inhibitory tyrosine-based switch motif (ITSM). Earlier mutagenic studies have shown that activated switch motif (ITSM) is required for the inhibitory effect of PD-1 on active T cells (21). Like other inhibitory co-receptors PD-1 is expressed by activated T cells, along with B cells, monocytes, NK cells, DCs, TILs, and activated T-regs, facilitating the proliferation of T-reg and thus, impeding immune response (22,23).

The PD-1 receptor has two ligands, PD-L1 (B7-H1 or CD274) and PD-L2 (B7-DC or CD273), which are shared by a co-inhibitory receptor CD80 (B7-1) (24,25). PD-L1 is expressed upon resting T cells, B cells, macrophages, DCs, pancreatic islet cells and endothelial cells. On the other hand, PD-L2 has restricted tissue distribution and is expressed only on antigen-presenting cells (APC). These differences in tissue distribution pattern suggest that

these two molecules have separate function in immune modulation. This restricted expression of PD-L2 to macrophages and DC is in line with its role in regulating T-cell priming; in contrast, broadly expressed PD-L1 is involved in protecting peripheral tissues from excess of inflammation and autoimmune pathologies. PD-L1 has been found to be overexpressed in a wide variety of cancers *viz*. melanoma, glioblastoma, multiple myeloma, leukemias, and gastric, renal cell, bladder, hepatocellular, cutaneous, and NSCLC (26-32). PD-L1 is heterogeneously expressed in cancer and is found to be located in the cytoplasm and plasma membrane of cancer cells (33). PD-L2 is also expressed by various tumor cells, including lung adenocarcinomas (34).

PD-L1 expression is induced by various proinflammatory molecules such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and cytokines like IL-10. The tumor microenvironment has activated T helper cells, which produce IFN-γ and TNF-α, and tumor stromal cells secreting VEGF and GM-CSF, thus upregulating PD-L1 expression in tumor cells and facilitating immune suppression. This phenomenon has been named "adaptive immune resistance" (33). IFN-γ-induced PD-L1 expression in human cholangiocytes is shown to be regulated microRNA-513 (35). mir-531 is complementary to the 3'-UTR of PD-L1 mRNA, thus it prevents its translation. In addition, expression of PD-L1 is also regulated by phosphatase and tension homolog deleted on chromosome ten (PTEN), a tumor suppressive gene. PTEN is often mutated in tumor cells, resulting in the activation PI3K/AKT pathway and downstream effector mTOR-S6K1 signaling cascade, which causes increased translation of PD-L1 mRNA and high expression of PD-L1 protein in the cancer cells (36,37). In melanoma, the higher levels of PD-L1 protein in tumor cells were found to be correlated to the CD8+ T-cells (38).

In tumor cells as well as in T cells, PD-L1 can act both as a ligand and receptor. PD-L1, in absence or unavailability of its receptor PD-1, is capable of stimulating T cells (39,40). Stimulatory signal for activating T cell is received through binding of CD28 surface molecule to CD80 and CD86 molecules expressed on the APCs, on the other hand, interaction of CTLA-4 with these molecules down regulates T cell activity (41). However, T cells can also express CD80 and its docking with PD-L1 could induce T cell tolerance, and at this point CD80 acts an additional T cell counter receptor for PD-L1 with inhibitory function (24).

PD-1/PD-L1 signaling pathway

Under normal physiological conditions, PD-1 regulates the activity of effector T-cells in peripheral tissues in response to infection. This is a critical step in protecting human body against tissue damage upon the activation of immune system. PD-L1 and PD-L2 expressed on the APC surface exert suppressive action via T-cell receptors. The binding of PD-L1 or PD-L2 leads to recruitment of tyrosine phosphatases generating an inhibitory signal that blocks downstream effects of PI3K/Akt signaling resulting in cell cycle arrest and suppressed T-cell activation (21,42,43). However, when cancer cells are attacked by immune system they start overexpressing PD-L1 and PD-L2, which bind to PD-1 receptors on T-cells suppressing them and in the process resulting in tumor immune escape (44).

In dynamic tumor microenvironment, PD-1 and its ligand PD-L1 down regulate T-cell response by various mechanisms. Recently, Duraiswamy & coworkers showed that overexpression of PD-L1 on tumor cells, tumor-associated macrophages, DCs, and MDSCs positively correlated with the exhaustion of TILs in tumor (45). PD-L1 down regulates phospho-Erk and increases PTEN causing inhibition of Akt/mTOR/S6 signaling pathway, hence, promoting generation of induced T-regs, which restrain the activity of effector CD8 T cell (23). Also, earlier reports show that PD-1 induces cell death in activated T cells via inhibiting PI3K activation leading to down regulation of anti-apoptotic protein Bcl-xL (46). Anti-PD-L1 antibody has been shown to decrease the apoptosis of T cells, and could help in achieving tumor immunity. In addition, PD-1 could also inhibit phosphorylation of PKC-θ, which is required for production growth stimulatory IL-2, thus resulting in cell cycle arrest and blocking T cell proliferation (47).

PD-L1 is overexpressed in various malignancies *viz*. melanoma, glioblastoma, and renal, gastric, head and neck squamous cell, colon, pancreas, breast, cervical, ovarian, and lung carcinoma (31,32,48-57). PD-L1 protein expression is a poor prognostic biomarker in NSCLC and found to be associated with tumor differentiation (58). Studies have shown that lung cancer patients with EGFR mutations have higher levels of PD-L1 expression than the wild-type samples and high PD-L1 expression is associated with the presence of EGFR mutation (59). In lung cancers not harboring EGFR mutation, the PD-L1 over expression

conveys poor prognosis (60). Also, a recent study revealed a direct link between tumor-related genes and imbalance of immune regulation. The authors showed that either *EML4-ALK* fusion gene or activating mutations of the EGFR upregulated PD-L1 expression in NSCLC cell lines by activating PI3K-AKT and MEK-ERK signaling pathways (61). There was also a direct correlation between the levels of EML4-ALK and PD-L1 expression in NSCLC tissue specimens.

Agents targeting PD-1/PD-L1

Currently, several immune-oncology agents targeting PD-1/PD-L1 are being developed. These novel promising immune checkpoint blockers have shown benefits in recent clinical trials, including the NSCLC patients.

As described above, PD-1 is an immunoregulatory receptor that is expressed by activated T cells (62). Although not all the cells expressing PD-1 are exhausted, postulating a theory that blocking PD-1 can restore the function of T cells (63). Nivolumab (BMS-936558 or MDX1106b) is a human IgG4 antibody against PD-1, and lacks detectable antibody-dependent cellular toxicity (ADCC). In phase I clinical trials Nivolumab showed remarkable regression in various tumors, including NSCLC (64), and in a recent study, previously treated metastatic squamous-cell NSCLC patients had a significantly better overall survival, response rate, and progression-free survival with Nivolumab than with Docetaxel (65). In March 2015, by the United States Food and Drug Administration (FDA) approved nivolumab to be used in treating patients with metastatic squamous NSCLC that progressed on or after platinum-based chemotherapy.

Pembrolizumab (MK-3475) is another highly selective anti-PD-1 humanized monoclonal IgG4 kappa isotype antibody that contains mutation at C228P designed to prevent Fc-mediated cytotoxicity. It can disrupt the engagement of PD-1 and PD-L1, resulting tumor recognition by cytotoxic T cells. In a recent phase-I trial, Pembrolizumab showed antitumor activity and had an acceptable toxicity profile in patients with advanced NSCLC (66).

Another strategy of attenuating PD-1 and PD-L1 signaling cascade is by anti-PD-L1 antibody binding with PD-L1 molecules. Targeting PD-L1 might also result in less treatment-related toxicity partly by instigating selective immune response in the tumor micro milieu. BMS-936559/MDX1105 and MPDL3280A are anti-PD-L1 monoclonal antibodies reacting specifically with PD-L1, and preventing its docking with PD-1 and CD80. BMS-936559/MDX1105 is a high affinity, fully humanized IgG4 antibody, whereas MPDL3280A is an engineered, human monoclonal IgG1 antibody with modified Fc component so as not to activate ADCC. In a multicentric, dose-escalation phase I trial in advanced solid tumors that included 75 NSCLC patients, 6–17% of objective response rate (ORR) and prolonged stabilization of disease (12–41% at 24 weeks) were observed (67). In a phase I trial of MPDL3280A, 175 patients (with 53 NSCLC cases) were enrolled and had a 21% ORR with 39% having stable disease (68).

Predictive biomarkers for immune-modulatory agents

With the development of targeted and more personalized therapies, there is a need to identify predictive biomarkers with which to select patients for treatment with a particular agent. In recent years, there have been various reports exploring PD-L1 expression as a criterion for selecting anti-PD-1/PD-L1 therapies (69).

The initial phase I Nivolumab trial in 296 patients reported 18% ORR in NSCLC patients reported (17 of 76 patients), 28% among patients with melanoma (26 of 94 patients), and 27% among patients with renal-cell carcinoma (9 of 33 patients). Immunohistochemical analysis on pretreatment tumor samples revealed that patients with PD-L1 positive (≥5% tumor cells with PD-L1 expression) tumor specimens had an excellent 36% ORR. On the other hand, patients with PD-L1 negative tumor specimens did not have any objective response (70). Similarly, in a phase I study with anti-PD-L1 antibody (MPDL3280A) across various solid tumors, responses (as evaluated by RECIST, version 1.1) were observed in patients with tumors expressing high levels of PD-L1, especially when PD-L1 was expressed by tumor-infiltrating immune cells (68). However, in a randomized, multicentric, phase-3 study that compared Nivolumab monotherapy with Docetaxel monotherapy in patients with advanced squamous-cell NSCLC in whom the disease progressed during or after one prior platinum-containing chemotherapy regimen authors found that the PD-L1 expression was neither prognostic nor predictive of therapeutic benefit (65). These conflicting reports call into question the potential role of PD-L1 as a predictive biomarker to select a patient subgroup for anti-PD-1/PD-L1 therapy.

These contradicting observations of PD-L1

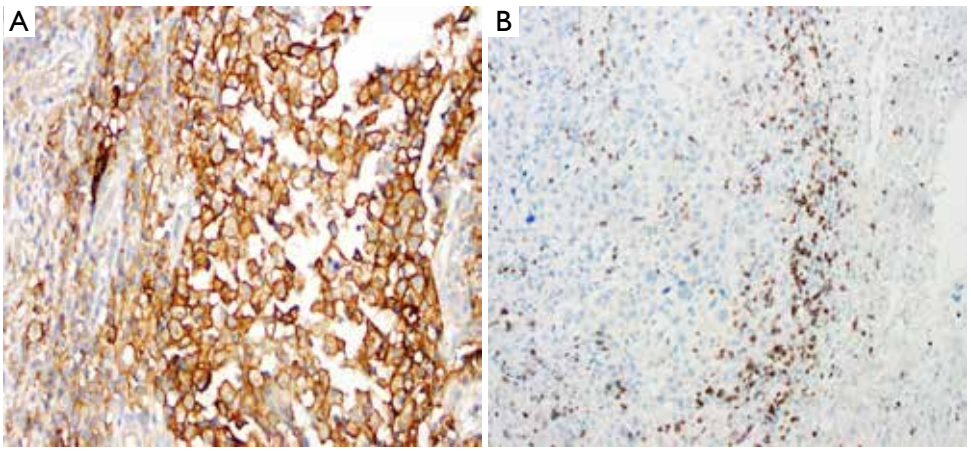

Figure 1 Immunohistochemical labeling for programmed death ligand-1 (PD-L1) performed with an anti-PD-L1 {clone EPR1161 [2]} & PD-1 [clone (NAT105)] from Abcam (Cambridge, MA, USA) with HIER using citrate buffer & LSAB method (Dako, Carpinteria, CA, USA). (A) Melanoma cells showing strong, uniform reaction in tumor cells (magnification, ×200); (B) PD-L1 negative in tumor cells, there is a positive reaction in immune cells in the tumor stroma and TILs (magnification, ×200). anti-PD-L1, anti-programmed death-1; HIER, heat-induced epitope retrieval; LSAB, labeled streptavidin biotin; TILs, tumor-infiltrating lymphocytes.

immunoexpression and possible correlation with tumor response may be attributed to the use of various antibodies for detecting PD-L1 by immunohistochemistry (IHC) in clinical trials. Initial report from Topalian used a murine antihuman PD-L1 monoclonal antibody 5H1 (70); Herbst *et al.*, used a an anti-human PD-L1 rabbit monoclonal antibody clone SP142 from Ventana, Tucson (68), whereas, Brahmer and coworkers used a rabbit monoclonal, antihuman PD-L1 antibody (clone 28–8, Abcam) (65). In an in-depth review by Teixidó *et al*. they consolidated an extensive list of PD-L1 antibodies used to-date for immunohistochemically detecting PD-L1 expression in various studies (69). All three studies examined PD-L1 expression in formalin-fixed paraffin-embedded (FFPE) tissue sections, however tissue fixation, epitope stability and processing could impact the outcome of immunohistochemical reactions (71).

Whereas frozen specimens can be stained very specifically, FFPE material could result in weaker staining and high background. Such observations have been confirmed by others using PD-L1 monoclonal antibody 5H1 (33,72). Unmasking of the epitopes that are masked by protein linkage during formalin fixation is performed via heat-induced epitope retrieval (HIER) using buffer solutions [citrate buffer (pH 6), ethylenediaminetetraacetic acid (EDTA) buffer (pH 9), and Tris buffer (pH 10)], which includes high-temperature heat-induced cleavage of the protein—protein cross-links produced during fixation of tissues in neutral-buffered formalin (73). In various studies published so far there is limited information on methodology for PD-L1 staining; specifically whether a low- or high-pH antigen retrieval buffer was used is unclear. However, it has been documented that HIER with a citrate buffer (pH 6) can produce results superior to those obtained with buffers with a higher pH (74).

In our laboratory, we have performed immunohistochemical analysis of PD-L1 protein expression in FFPE sections of melanoma lesions using a rabbit monoclonal antibody against PD-L1 {clone EPR1161 [2] Abcam, Cambridge, MA, USA} at dilution 1:100, after HIER with citrate buffer (pH 6). We have observed a predominantly membranous pattern along with occasional cytoplasmic staining (*Figure 1A*). For PD-1 immunostaining, after HIER using citrate buffer (pH 6), we have used mouse monoclonal antibody against PD-1 of human origin [clone (NAT105); Abcam, Cambridge, MA, USA] at dilution 1:250, and have observed only membranous staining in the lymphocytes (*Figure 1B*). For both these antibodies we have used labeled streptavidin biotin (LSAB) method (Dako, Carpinteria, CA, USA), which compared to ABC method results in less background staining.

Furthermore, even if the immunohistochemical study is correctly performed, it may be difficult to determine a cutoff that defines a clinically significant positive and predictive value. Furthermore, the goal is to determine which patients will likely benefit of the treatment. Although most studies have regarded PD-L1 membranous labeling

of the tumor cells as the most significant one, PD-L1 expression can be detected by IHC in both tumor cells and in immune effector cells (tumor infiltrating immune cells) (17,70,75).

Regarding evaluation of the immunohistochemical slides, there may be different cut-offs, such as ≥1%, ≥5%, or ≥10% of cells (68); ≥5% cells (70); or 1%, 5% or 10% cells in at least 100 evaluable tumor cells (65). A possible pitfall in some of these studies is the fact that PD-L1 is heterogeneously expressed in tumor cells and thus examination of tissue microarrays or even just a small biopsy of a large tumor may not project an accurate picture of PD-L1 pattern of expression.

In addition to evaluating PD-L1 expression, other biomarkers that play a role in antitumor response educed by immunotherapy may also be identified through genetic analysis of tumor cells. In patients treated with MPDL3280A, high expression of CTLA4 (an important regulator during T-cell expansion) in pre-treatment specimens was observed to correlate with response to therapy (68).

Conclusions

Based upon the studies reported so far, use of PD-L1 expression as a predictive biomarker for anti-PD-1/PD-L1 immunotherapy remains a possible method for selection or exclusion of patients for treatment (28). However, due to the existence of various available antibodies for detecting PD-L1 expression with different scoring systems, epitope stability, usage of varying sample types *viz.* small biopsies, cytology specimens and large tumor samples, and intra- and inter-laboratory reproducibility, it remains to be determined the optimal, standardized method for clinical trials.

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Footnote

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Cancer stem cells and immunoresistance: clinical implications and solutions

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Abstract: Tumor cells can be contained, but not eliminated, by traditional cancer therapies. A cell minor subpopulation is able to evade attack from therapies and may have cancer stem cell (CSC) characteristics, including self-renewal, multiple differentiation and tumor initiation (tumor initiating cells, or TICs). Thus, CSCs/TICs, aided by the microenvironment, produce more differentiated, metastatic cancer cells which the immune system detects and interacts with. There are three phases to this process: elimination, equilibrium and escape. In the elimination phase the immune system recognizes and destroys most of the tumor cells. Then the latency phase begins, consisting of equilibrium between immunological elimination and tumor cell growth. Finally, a minor attack-resistant subpopulation escapes and forms a clinically detectable tumor mass. Herein we review current knowledge of immunological characterization of CSCs/TICs. Due to the correlation between CTCs/TICs and drug resistance and metastasis, we also comment on the crucial role of key molecules involved in controlling CSCs/TICs properties; such molecules are essential to detect and destroy CSCs/TICs. Monoclonal antibodies, antibody constructs and vaccines have been designed to act against CSCs/TICs, with demonstrated efficacy in human cancer xenografts and some antitumor activity in human clinical studies. Therefore, therapeutic strategies that selectively target CSCs/TICs warrant further investigation. Better understanding of the interaction between CSCs and tumor immunology may help to identify strategies to eradicate the minor subpopulation that escapes conventional therapy attack, thus providing a solution to the problem of drug resistance and metastasis.

Keywords: Cancer stem cell (CSC); immunoresistance; immunotherapy

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Introduction

Tumor immunosurveillance

Cancer is caused by an accumulation of genetic alterations in cells which drive activation or overexpression of proteins that promote cell cycle arrest and cell survival, while other proteins that promote cell cycle arrest or cell death are inactivated or downregulated (1). In normal circumstances, most of these lesions are repaired or the mutated cells are eliminated by control mechanisms such as DNA repair enzymes, tumor suppressor genes (2) and the immune system (3). Thus growth of tumor cells is prevented and innate immunity constitutes a first line of defense. Stress induces upregulation of ligands that activate natural killer (NK) cell receptors (4) and other immune stimulatory surface molecules that recognize and eliminate tumor cells.

This response can activate an adaptive immune response against antigens specifically expressed by lysed tumor cells and lead to T cell-dependent tumor control. Key molecules for tumor immunosurveillance are interferon-gamma (5), interleukin-12 (IL-12) (6), perforin (7), TRAIL (8), DR4 and DR5 (9) and the recombination activating genes RAG1 (10), and RAG-2 (5). RAG1 and RAG-2 are required for cell development, as is the T cell receptor (11,12). Loss of any of these molecules results in more frequent or faster spontaneous or carcinogen-induced tumorigenesis. The ability of cells to evade destruction by the immune system is thus recognized as a hallmark of cancer (2).

Cell immune surveillance evasion

The immune system is able to maintain tumor growth in a dormant state for decades without completely eradicating all the malignant cells. Certain factors may reduce the ability of the anti-tumor immune system to detect and eliminate malignant cells: pre-established tolerance resulting from non-recognition of tumor antigens (13), generation of less immunogenic tumor cell subclones and immunosupressor molecules such as cytokines or hormones that cause NK and T cell suppression in the tumor microenvironment (14). Lower levels of activatory and/or higher levels of inhibitory NK cell receptor ligands may allow some malignant cells to survive (15). Aggressive tumors are often characterized by low levels of classical human leukocyte antigen (HLA) class I molecules. People with immune system deficiencies such as human immunodeficiency virus (HIV) (16), or who have undergone an organ transplant (17), and the very elderly run an increased risk of developing cancer (18).

Cancer stem cells (CSCs) hypothesis

Tumors are composed of heterogeneous cell subpopulations, defined by two different theories: the stochastic or clonal evolution model, and the hierarchical or CSC model. These theories appear to be mutually exclusive but new data suggest that neither should be discounted (19). In the stochastic model, all tumor cells are biologically equivalent, with a similar capacity for self-renewal and formation of new tumor cells. Cell heterogeneity arises from subclonal differences resulting from genetic and/or epigenetic changes during cancer development. In the hierarchical model, only a cell subpopulation—also known as tumor initiating cells (TICs) (20)—is able to initiate tumor growth. The hierarchical hypothesis defines CSCs as a minority cell tumor subpopulation endowed with properties such as self-renewal, differentiation and multi-potency. CSC-like properties may also be a function of cell type origin, signals from the stromal microenvironment, accumulated somatic mutations and stage of malignant progression (21). These cells display resistance to chemotherapy (22), radiotherapy (23) and immunotherapy (24) and are TICs (4).

Several mechanisms, such as quiescence, are involved in chemoresistance (22). Certain drug-resistant proteins also make stem cells more resistant to toxins that kill their terminally differentiated counterparts (25). For example, resistance is dependent on IL-4 signaling, since up-regulation of IL-4 may result in resistance to apoptosis (26). In addition, CSCs/TICs that have undergone an epithelial-mesenchymal transition (EMT) appear to be more resistant to chemotherapy (27). An increase in aldehyde dehydrogenase (ALDH) activity in these cells seems able to mediate resistance to some chemotherapic agents (28). B-cell lymphoma-2 (BCL-2) protein and its family members (29) also constitute another mechanism of chemoresistance. Therefore, CSCs/TICs possess different mechanisms of resistance to several therapies.

Exact characterization of markers that allow identification of CSCs/TICs in different tumors is still not possible, since no markers have been reported as being unique to CSCs/TICs. Markers such as CD166 have been defined for several tumors. For example, CD166 is a marker of CSCs/TICs in non-small cell lung cancer (NSCLC) (30). The diversity of markers associated with CSCs/TICs may be due to the existence within the tumor tissue of different subpopulations endowed with stem cell features but also with distinct biological properties (31) reflecting differences in patients' genetic backgrounds and intra-and/or inter-cancer heterogeneity of the primary tumor (32).

CSCs/TICs and the immune system

Immune system and elimination of CSCs/TICs

The process by which the immune system detects and interacts with tumor cells, both before and after clinical detection of the tumor, is known as tumor immunoediting. This process has three phases: elimination, equilibrium and escape (33). In the elimination phase, the innate and the adaptive immune system recognize and destroy most of the tumor cells. However, some malignant cells escape and a latency phase begins, consisting of equilibrium

between immunological elimination and growth of tumor cells that may persist for months, years or decades (34). During this period, the cells suffer genetic and epigenetic changes and some generate new immunogenic peptides, enabling the tumor to eliminate these cells. However, some of these changes generate a poorly immunogenic stem cell subpopulation that circumvents immune recognition and also these cells may manipulate the immune system to promote their own growth (35). However, the lack of a favorable microenvironment, and a low rate of cell division, still prevents the formation of a tumor mass (36). Finally, the less immunogenic CSCs/TICs, and the more aggressive clones, are able to form a clinically detectable tumor mass and initiate the escape phase. The reasons for this are as follows: (I) CSCs/TICs can produce immunosuppressive molecules that attenuate the immune system (34); (II) CSCs/TICs recruit cells that suppress the immune system (37); (III) immunology tolerance due to loss of tumor antigen expression, loss of antigen processing and presentation machinery, down-regulation major histocompatibility complex (MHC) class-I (MHC I) expression, and inhibition of co-stimulatory or MHC II molecule expression on antigen presenting cells (APCs) due to genetic alterations (38). Also, the immune system may be weakened by illness, aging or therapeutic immunosuppression. Certain signaling pathways, such as Notch, Wnt and Hedgehog, are able to promote CSC/TIC escape (39).

Immunological characteristics of CSCs/TICs

The capacity of CSCs/TICs to present tumor antigens to T cells for immune recognition or to elicit immune response is determined by expression of antigen presentation molecules, such as MHC-I and MHC-II, as well as co-stimulatory (e.g., CD80, CD86) and co-inhibitory molecules [e.g., cytotoxic T-lymphocyte antigen 4 (CTLA4), B7-H2, B7-H3, programmed death receptor 1 (PD-1)/-1L] (where co-stimulatory molecule expression is negative for these cells and expression of co-inhibitory molecules is up-regulated) (40). CSCs/TICs subsequently show down-regulation of MHC-I and lack MHC-II molecule expression, resulting in downregulation of low molecular weight protein (LMP) antigen processing systems, a transporter associated with antigen processing (TAP), and beta macroglobulin which elicits escape from immune system attack (41).

CSCs/TICs have been shown to secrete cytokines such as transforming growth factor beta (TGF-β), IL-10 and IL-13 *in vitro* (42). In glioblastoma, CSC/TIC survival has been found to be dependent on secretion of associated angiogenic factors such as vascular endothelial growth factor (VEGF), macrophage-chemoattractant protein-1 (MCP-1), macrophage inhibitory factor (MIF), growth related oncogene alfa (GROα) and ecotaxin (43). Also, TGFβ, IL-6 and IL-8 expression are downregulated in CSCs/TICs (43). In addition, stromal fibroblasts of the tumor microenvironment may be involved in regulating CSC/TIC generation by release of CCL-2 (44). Breast cancer and glioblastoma CSCs/TICs secrete more TGFβ than normal cancer cells (45). Colon CSCs/TICs secrete IL-4, which promotes drug resistance and inhibits anti-tumor immune responses (46). CD200 is also expressed in CSCs/TICs and plays an important role in immune escape (47).

Anti-apoptotic molecules like bcl-2, bcl-xL and survivin protect cells against chemotherapy as well as conferring increased resistance to apoptosis-inducing immune effectors like T or NK cells (48). In a similar manner, the PI3K/Akt pathway mediates chemoresistance and tumor immune escape (49). HER2 interferes with antigen processing and presentation and is key to maintenance of CSCs in luminal breast cancer (50). In summary, CSCs/TICs express soluble and membrane-bound molecules that modulate immune responses and protect cells from immune system attack.

The STAT3 pathway plays an essential role in tumor-mediated immunosuppression by inhibiting macrophage activation (51). STAT3 pathway also reduces the cellular cytotoxicity of NK cells and neutrophiles as well as expression of MHC II, CD80, CD86 and IL-12 in dendritic cells (DCs), rendering them unable to activate T cells and initiate antitumor immunity (52). In addition, STAT3 regulates transcription of immunosuppressive factors such as IL-10, VEGF, PGE_2 and TGF-β (53). It has been shown that STAT3 signaling is up-regulated in glioma CSC/TICs, and growth and self-renewal of this subpopulation is dependent on this pathway. CSCs/TICs also secrete some factors that induce STAT3 phosphorylation in immune cells (54).

Tumor-associated antigens (TAAs) expressed by CSCs/TICs

CSCs/TICs express TAAs, which characterize their condition of "stemness" and can be recognized by T cells. TAAs are classed as different subgroups of molecules (41,55) as follows:

(I) Differentiation antigens from which the tumor derives and which could also be expressed by normal cells, i.e., carcino-embryonic antigen (CEA)

in colon cancer, mucin-1 (MUC-1) in breast cancer, and gp100 and tyrosinase in melanoma (56);

(II) hTERT and surviving antigens, and other apoptosis-inhibitory proteins expressed by non-stem cancer cells in addition to subsets of normal cells (57);

(III) Cancet-testis (CT) antigens such as Melanoma-associated-antigen-A3 (MAGE-A3) and A4 and NY-ESO1 expressed in normal cells, tumor cells and CSCs/TICs (57);

(IV) Mutated antigens deriving from somatic point mutations in tumor cells that can result in entirely new epitopes recognizable by the immune system (58).

In melanoma, the CSC/TIC subpopulation that express ATP-binding cassette sub-family B member 5 (ABCB5) elicits tumor cell dissemination through mediation of chemotherapy resistance, has low levels of lineage-related and CT antigens (59). However, the $CD133^+$ melanoma cell subpopulation has high expression of NY-ESO1 cancer testis antigen as well as susceptibility to specific T cells (60). The TAA DDX3X has been found in $CD133^+$ CSCs/TICs in melanoma and many cancers, conferring immunogenicity on these cells and their ability to induce T-cell dependent protection against murine cancer growth *in vivo* (61). In contrast, the $CD271^+$ CSC/TIC melanoma subpopulation is deficient in the expression of both lineage-related and CT antigens, making their removal by immune T cells difficult. This has been correlated with progression and metastasis of these cells. As such, melanoma cells offer a good example of multiple CSC/TIC subpopulations with different antigen expression patterns (62).

None of these potential TAAs seem to be a specific marker of CSCs/TICs since they may also be expressed in both tumoral and normal cells. However, T cell responses against TAAs are expressed by CSCs/TICs, such as IL-13Rα2, SOX2 and CD133 in gliomas (63), CEP55 and COA-1 in colorectal cancer (CRC) (64) and epithelial cell adhesion molecule (EpCAM) in retinoblastoma (65). A possible exception is TAAs resulting from somatic point mutations of tumor cells and their CSCs (66).

Immune targeting of lung CSCs/TICs

Introduction

There is a strong relationship between resistance to conventional therapies and intrinsic mechanisms of CSC/TIC resistance to chemo or radiotherapy. Direct targeting of CSCs/TICs or specific signaling pathways responsible for resistance can improve treatment benefit (67). Until recently, in contrast to tumors like melanoma, lung cancer was not thought to be immunogenic. Several immunotherapies, such as IL-2, interferon and bacille Calmette-Guerin, have been tried but have not proved successful to control the immune system in NSCLC patients. Therefore, immunotherapy for NSCLC was considered unsuccessful (68). However, immunotherapeutic approaches involving both stimulation of immune responses and inhibition of immune checkpoints have now been tested and could be combined with chemotherapy or targeted therapies with demonstrated efficiency in lung cancer (*Figure 1*). A body of evidence now suggests lung cancer is immunogenic. Lung cancer cells release growth factors, interleukins, cytokines and prostaglandins that inhibit T-cell response to the microenvironment, and also has been described that increased tumor-infiltrating lymphocytes (TILs), NK cells, DCs, cytotoxic T lymphocytes (CTLs) and T helper cells are associated with improved survival in NSCLC (69). Also, a high ratio of effector T-cells to regulatory T cells (T-reg) is associated with improved long-term survival (70). In addition, increased immunosuppressive T-regs as a proportion of total TILs are associated with poorer survival in lung cancer (69). MHC class I expression is reduced in NSCLC and these tumors can therefore escape routine antigen processing (71).

Immunotherapy tends to produce durable responses in small subpopulations of patients. The challenge currently facing investigators is to identify biomarkers predictive of response. Good examples so far are CTLA4 and PD-1 and its ligand (72). Immune targeting of stem cells carries some risks, one obvious one being that pathways are shared with normal adult stem cells, and autoimmunity could carry toxicity to these normal cells. Therefore, it is crucial to identify markers exclusive to CSCs (73). Other obstacles could also limit immune responses, such as a variety of defense mechanisms like soluble mediators TGF-β and COX-2 which make prostaglandin E, IL-10 and arginase. Also defensive molecules such as Fas ligand, B7-H1, nonconventional HLA molecules, lack of MHC class I and recruitment of suppressor type cells (74). Very low levels of expression of these molecules limit detection and elimination of CSCs/TICs.

Cancer cells express many antigens that can be recognized and presented to T cells, leading to T cell activation and elimination of these tumoral cells. This T cell immune response is modulated by negative regulatory

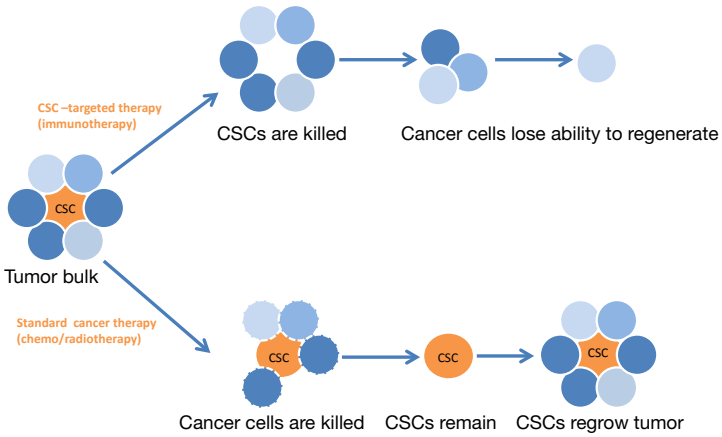

Figure 1 Heterogeneous subpopulation of cancer cells could be treated with standard therapy, as chemo- or radio-therapy. This treatment eliminates cancer cells but not CSCs and tumor grows back. If the cancer cells are treated with CSC-targeted therapy, as monoclonal antibodies or vaccines, the immune system could be stimulated or immune checkpoints inhibited, and then CSCs are killed and tumor loses its ability to generate new cancer cells. CSC, cancer stem cell.

Table 1 Immune checkpoint blockade

Compound	Target
Ipilimumab	CTLA4
Tremelimumab	CTLA4
Nivolumab	PD-1
Pembrolizumab	PD-1
BMS-936559	PD-L1
MPDL3280A	PD-L1
MEDI4736	PD-L1
Bec2	GD3
Bevacizumab	VEGF
Urelumab	CD137
TRX518	GITR
Anti-OX40	OX40
Anti-CD40	CD40
Solitomab	EpCAM
Anti-CD133	CD133
Lirilumab	KIR
BMS-9896016	LAG-3
Racotumomab	N-glycolil-GM3 ganglioside

CTLA4, cytotoxic T-lymphocyte antigen 4; PD-1, programmed death receptor 1; PD-L1, programmed death ligand 1; VEGF, vascular endothelial growth factor; GITR, glucocorticoid-induced tumor necrosis factor receptor; EpCAM, epithelial cell adhesion molecule; KIR, killer cell immunoglobulin-like receptor; LAG-3, lymphocyte-activation gene 3.

molecules such as the immune checkpoint molecules CTLA-4, PD-1, killer cell immunoglobulin-like receptor (KIR) and lymphocyte-activation gene 3 (LAG3); these molecules prevent overstimulation of immune responses. The T cell immune response could be also modulated by co-stimulatory molecules such as glucocorticoid-induced tumor necrosis factor receptor (GITR), OX-40, CD28 and CD137 (75). Deregulation of these molecules in the tumor leads to tolerance of the tumor by the immune system and cancer cell escape from surveillance. A description of the different compounds tested to target these regulatory molecules follows (*Table 1*).

Immune checkpoint blockade

Immune checkpoints are inhibitory pathways crucial for maintaining self-tolerance and to escape to immune system control by the tumor (76). It has observed that inhibitory ligands and receptors are usually overexpressed in cancer cells or their microenvironment (77). Inhibition of these immune checkpoints releases the brakes on the immune system, resulting in antigen-specific T-cell responses. Such inhibition of immune checkpoints relies on the presence of TILs. Stimulation of TILs and/or modulation of the tumor microenvironment could weaken immune responses (78). In lung cancer, targeting the immune checkpoint molecules, CTLA4, PD-1 and its ligand PD-L1 has achieved promising and durable responses but it

remains unclear why some patients have only transient or no response (79). One strategy is to target CSCs/TICs with monoclonal antibodies targeting antigens that are differentially overexpressed in these cells. These could be used alone as unmodified antibodies to allow antibody-dependent cytotoxicity (ADCC) to occur, or used with radioisotopes, chemotherapy, cytokines or enzymes to target cancer. A problem of this treatment is that stem cells could escape the cytotoxic effect of specific antibodies by decreasing expression of surface antigen, developing chemotherapy resistance or acquiring multiple mutations. Therefore, antibody treatment is used in combination with conventional cancer therapies (80).

CTLA4

CTLA4 is an immunomodulatory molecule expressed in T cells which plays a role in regulating T-cell activity at early stages of activation; its expression on T cells increases after exposure to an antigen. Binding of the CTLA-4 receptor to CD80/86 expressed on APCs has a co-inhibitory effect on T cells. By competing with the CD28 molecule for the same ligands, albeit with a higher binding affinity than CD28, CTLA-4 inhibits T-cell activation (68). Negative signals are delivered to T cells upon binding to APC CD80/CD86 molecules via CTLA4, T cell function is inhibited and T cells can then be eliminated via apoptosis. Lung cancer could stimulate abnormal expression of CTLA-4 in T cells and these T cells exhibit an anergic phenotype (81). There are currently several clinical trials in lung cancer with human monoclonal antibodies against CTLA4 like ipilimumab or tremelimumab. To date, the response rate is low but these responses are more durable than with cytotoxic therapies (82).

Ipilimumab is a human monoclonal antibody that blocks binding of CTLA-4 to its ligand. As a single agent it has virtually no effect (83) but does seem to provide modest benefit in NSCLC and small cell lung cancer (SCLC) patients in combination with chemotherapy. A phase II study of chemotherapy, paclitaxel and carboplatin with and without ipilimumab in stage IV NSCLC showed a significant improvement in progression-free survival (PFS) when ipilimumab was given after chemotherapy (5.7 *vs.* 4.6 months) (84). Patients with squamous histology showed better response than non-squamous histology. Now are several clinical trials ongoing. A phase III trial is currently comparing ipilimumab to placebo in SCLC patients receiving platinum and etoposide, and another phase II is comparing ipilimumab to pemetrexed in non-squamous NSCLC. Ipilimumab is also being evaluated with the anti-KIR antibody BMS-986015 that recognizes KIR in NSCLC, castration-resistant prostate cancer (CRPC) and melanoma (85). These antibodies must be used carefully as they can cause autoimmunity and other severe side effects that limit their use (86). Tremelimumab, which also targets CTLA4, has been tested as maintenance therapy compared with observation in patients with stable or responding disease after first line chemotherapy, however, no improvement in PFS was seen (87).

PD-1

PD-1, like CTLA-4, is a member of the CD28 family. PD-1 is expressed in T cells and inhibits their survival, proliferation and immune function through interaction with its ligands PD-L1 and L2. PD-1 is also expressed in B cells and in some myeloid cells (88). Interactions between PD-1 and its ligands attenuate immune responses (89) and serve to protect tumor cells from cytotoxic T cells since T cells become triggered for apoptosis upon signal transduction with PD-1 family proteins (90). Clinical trials with humanized monoclonal antibodies against PD-1 have shown good antitumor activity in subsets of patients with metastasis disease with a good safety profile (80). Several PD-1 antibody trials are ongoing and one study has found a strong correlation between pretreatment tumor expression and responses (72).

Nivolumab, a human monoclonal antibody that binds to PD-1, has been tested in several clinical studies in NSCLC and in two trials specifically for primary squamous cell carcinoma (SQCC), either as a single agent or in combination with chemotherapy or ipilimumab (68). In other studies it was combined with anti-KIR antibody (91). In a phase I clinical study it was administered to 306 patients with different tumor types, including 129 NSCLCs. Overall response rate (ORR) of this study was 17% and the median duration of response was 47 weeks. Another 10% of patients showed stable disease for 6 months with median survival of 9.6 months. Thirty seven patients who received nivolumab at doses of 3 mg/kg showed 24% response rate and 14.9 months median survival (68). Ongoing phase III studies are comparing nivolumab *vs.* docetaxel in the second-line setting and a phase III first line trial of nivolumab *vs.* standard chemotherapy in PD-L1 positive metastatic NSCLC is currently recruiting (92). An ongoing phase I clinical trial is combining nivolumab plus ipilimumab with an ORR of 22% at time of interim analysis (93).

Another anti-PD-1 antibody similar to nivolumab, pembrolizumab (also known as MK-3475 or lambrolizumab) is a humanized IgG4 antibody that contains a mutation

at C228P designed to prevent Fc-mediated ADCC. In a phase I study, 38 NSCLC patients were treated with pembrolizumab, achieving 24% of lasting responses in previously treated patients. Pembrolizumab is now being examined in the relapsed/refractory setting (NCT01905657) and in combination with first-line chemotherapy (NCT01840579) (85,86).

PD-L1 (B7-H1)

Another therapeutic strategy is inhibition of PD-L1. PD-L1 is overexpressed in around 50% of NSCLC patients and is associated with poor prognosis. Its overexpression induces T-cell anergy and circumvents recognition and processing of tumor antigens by APCs (90). A potential advantage of this approach is lack of interference with T-cell PD-1 receptor interaction with APCs via other ligands, such as B7-H2 (94). A human anti-PD-L1 antibody, BMS-936559, has been tested in a phase I trial and showed promising clinical activity and good safety profile in NSCLC with partial response in 5 of 49 patients (68,95). Other antibodies in clinical development are MPDL3280A (RG7446), a human IgG1-kappa anti PD-L1 monoclonal antibody with a single amino acid substitution in its Fc region that docks with Fc receptors in circulating immune cells, thus preventing ADCC and inadvertent killing of bystander immune cells that also express PD-L1, such as activated T cells. In a phase I trial, 85 NSCLC patients received MPDL3280A as a single agent, with 23% best overall response and 24-week PFS of 46% (96,97). Another IgG1-kappa PD-L1 inhibitor is the antibody MEDI4736, engineered with a triple mutation in the Fc domain that also avoids ADCC as does MPDL3280A. MEDI4736 is currently being tested in a phase I clinical trial. In this study, of 11 NSCLC patients evaluated for efficacy, three achieved partial response, two showed stable disease and one had disease progression (68).

In conclusion, a few patients have good responses to anti-PD-L1 antibodies like nivolumab, MPDL3280A or MEDI4736, despite the absence of PD-L1 expression by immunohistochemistry. However, robust predefined cut-points or independent external validation methodology are not available in the literature. In addition, use of fresh or paraffin-embedded tumor samples could affect results in fresh samples due to the influence of cytokines, such as IFN-α, that upregulate PD-L1 expression (86,98).

GD3

GD3 is a cell surface ganglioside highly expressed in SCLC but not in NSCLC. Bec2/bacille Calmette-Guerin is an anti-idiotypic antibody that binds to the idiotype of the antibody against GD3. Therefore, Bec2/bacille Calmette-Guerin is thought to mimic GD3. In a phase III clinical trial in 515 limited stage patients, use of Bec2/bacille Calmette-Guerin showed no improvement in survival, PFS, or quality of life in the vaccination arm compared with control arm (median survival 16.4 *vs*. 14.3 months, respectively) (99,100). 1E10 is an anti-idiotypic antibody against Neu-glycosylated sialic acid ganglioside (NeuGc-GM3). It was used in clinical trials in SCLC and NSCLC, and a survival benefit of about 6 months was noted in those patients that developed immunity to NeuGc-GM3 (101).

Vascular endothelial growth factor (VEGF)

Bevacizumab is an anti-VEGF antibody that plays a role in tumor angiogenesis and inhibition of immune response by switching off the action of DCs. A phase III clinical trial in metastatic NSCLC demonstrated improved PFS and overall survival (12.5 *vs*. 10.2 months) (102).

Other immunotherapy compounds

CD137, GITR and OX40 are positive regulatory molecules of T cell immune responses. Now we describe some compounds that target these molecules.

Urelumab (BMS-663513) is a human IgG4 monoclonal antibody that targets CD137 receptor of the tumor growth factor alpha (TNFα) family and acts as co-stimulatory molecule of T cell activation. Urelumab activates a component of the TNF receptor expressed on the cell membrane of activated white blood cells, subsequently activating CD137-expressing immune cells and stimulating a cytotoxic T cell response against tumor cells. Clinical development in NSCLC has been stopped but is continuing in other cancers (NCT014712109) (85,86).

GITR is a member of the TNF receptor family. GITR co-stimulates $CD4^+$ and $CD8^+$ naïve T cells, leading to T cell proliferation and effector function (85,103). TRX518 is an anti-GITR antibody currently being tested in a phase I trial in melanoma (NCT01239134).

OX-40 (CD134) is also a member of the TNF receptor family. Like CD137 and GITR (101), OX-40 is a co-stimulatory molecule in activated T cells at sites of inflammation and regulates antigen-specific T-cell expansion, survival and cytokine production (IL-2, IL-4, IL-5, IFN-gamma) (104). In a phase I trial, 30 patients with solid tumors were treated with an anti-OX-40 antibody with tumor reduction in 12 patients and enhanced humoral and cellular immunity (75,105).

Table 2 Vaccines

Compound	Target
Stimuvax	MUC-1
Anti-NY-ESO1	NY-ESO 1
GSK1572932	MAGE-A3
CimaVax	EGF
Anti-WT-1	WT-1
Anti cyclophilin B	Cyclophilin B
Lucanix	TGF-β2
IDM-2101	CEA, p53, HER2, MAGE 2 and 3
Dendritic vaccine	p53
TG4010	MUC-1
Anti-IDO	IDO
GV1001	Telomerase

MUC-1, mucin-1; MAGE-A3, melanoma associated antigen A3; EGF, epidermal growth factor; WT-1, Wilms tumor antigen-1; TGF-β2, transforming growth factor beta 2; CEA, carcino embryonic antigen; IDO, indoleamine-2,3-dioxigenase.

CD40, a member of the TNF receptor family, is expressed in APCs and its ligand is expressed in T cells. Binding of both enhances APC ability to present antigens and activate T cells. Preclinical studies have demonstrated that anti-CD40 antibodies have the potential to suppress tumor growth and metastasis (106).

Racotumomab (1E10) is an anti-idiotype murine monoclonal antibody against the human monoclonal antibody for N-glycolil-GM3 ganglioside. N-glycolil-GM2 is a glycolipid present within gangliosides, sulfatides, and other antigens expressed in some solid tumors which seems to correlate with survival and suppression of immune activity in NSCLC. A phase III clinical trial (NCT01460472) is currently ongoing with a planned accrual of 1,018 participants (85).

EpCAM is a transmembrane glycoprotein overexpressed in most human carcinomas (107). Solitomab (MT110) is a single-chain bispecific T-cell engager (BiTE) antibody targeting EpCAM (108) which has been tested in dose escalation phase I clinical trials in patients with locally advanced, recurrent or metastatic lung cancer (109). CD133 is reported in CSC/TICs in lung cancer (110). A bispecific antibody against CD3 and CD133 has been designed to eradicate $CD133^+$ cancer cells (111).

KIR and LAG3 are negative regulatory molecules of T cell immune responses, like PD-1 and CTLA-4. Several monoclonal antibodies are designed to target these molecules.

KIR is a receptor on NK cells that downregulates NK cytotoxicity activity (86). Lirilumab (IPH2102), an anti-KIR human monoclonal antibody, was used in combination with nivolumab and demonstrated efficacy in preclinical models. A clinical trial in 32 NSCLC patients is ongoing (NCT01714739) as is another combining lirilumab plus ipilimumab in 20 NSCLC patients (NCT01750580) (86).

LAG3 (CD223) is a receptor expressed with PD-1 on tolerant T cells and T-regs which suppresses APC activation by binding with MHC II (112) and becoming an inhibitory molecule of T cell activation in the same manner as KIR. A clinical trial is ongoing with BMS-9896016, an anti-LAG3 monoclonal antibody, alone and in combination with nivolumab (NCT01968109) (86).

Vaccines

TAAs contain more than 70 proteins, including CT antigens such as MAGE-A3, and antigens like MUC-1 that are overexpressed in tumor cells. Using protein or peptide vaccines such as Stimuvax (tecemotide or L-BPLP25) and GSK1572932, TAAs can be targeted for subsequent killing of tumor cells (113).There are many TAAs expressed by tumors not identified, and to recognize them whole tumor vaccines were designed. Vaccines such as Lucanix can be harvested from the patient's own tumor (autologous) or from established cancer cell lines (allogeneic) and express many TAAs found in patient tumors, theoretically generating an immune response to the tumor (113).

Adaptative T-cell therapy is a passive strategy that involves the transfusion of T-lymphocytes to attack cancer cells in the patient. NY-ESO-1 is one such vaccines (113).

Vaccines currently in clinical trials in lung cancer (*Table 2*).

MUC1 is a highly glycosylated transmembrane protein overexpressed and abnormally glycosylated in many cancers including NSCLC (114). High levels of MUC1 could enhance immunosuppression and predict poor prognosis in patients with adenocarcinoma (115). Stimuvax is a 25-aminoacid MUC-1 peptide formulated into liposomes targeting MUC1 (116). Several clinical trials have already been performed, including a phase IIb study in stage IIIB and IV NSCLC (117). Median survival time in patients receiving Stimuvax was 17.2 *vs*. 13.0 months for those receiving best supportive care. Three year survival was 31% with Stimuvax *vs*. 17% for supportive care (118). Following this study, a phase III clinical trial in NSCLC

was carried out (START trial) in 1,513 patients with median overall survival of 25.6 months for patients treated with Stimuvax and 22.3 with placebo. Therefore, the trial did not achieve its primary endpoint of improvement in overall survival. However, analysis of treatment with Stimuvax plus chemotherapy and radiotherapy did show an improvement in median overall survival of 30.8 months compared to 20.6 months for placebo. Inspired by these results, a new phase III clinical study is currently ongoing (START 2 trial) with a primary end-point of overall survival in patients receiving Stimuvax plus chemotherapy and radiotherapy (113). Similar to the START trial, a phase III clinical trial in Asian NSCLC patients is ongoing (INSPIRE) comparing Stimuvax with placebo. In another phase III–IV trial, NSCLC patients were treated with Stimuvax plus bevacizumab following chemotherapy. In a stage III–IV trial, 16 of 65 patients showed a T-cell immune response and had median survival of 30.6 months compared to 13.3 months for best supportive care (85,119).

NY-ESO-1 is a fusion protein vaccine currently being tested in NSCLC (120). In a clinical trial with other tumors a measurable response rate of 66% (four of six patients) was reported in synovial cell sarcomas and 45% in melanoma (five of eleven patients) (120).

MAGE-A3 is an antigen present in about 35% to 55% of NSCLC patients. GSK1572932 is a recombinant DNA vaccine composed of MAGE-A3 and immunoadjuvant AS15. In a phase II clinical trial, 182 stage I and II patients were enrolled with a 27% improvement in time to progression and disease-free survival in patients receiving the vaccine. A phase III clinical trial is ongoing studying the combination of the vaccine with adjuvant chemotherapy in 2,270 NSCLC patients (121).

Mutations in the *epidermal growth factor receptor* (*EGFR*) gene are associated with cell proliferation, apoptosis, angiogenesis and metastasis. The epidermal growth factor (EGF) ligand is often overexpressed in lung cancer and its receptors frequently mutated (122). The CimaVax vaccine is a humanized recombinant EGF fusion protein that targets the EGF ligand circulating to prevent EGFR activation. Circulating anti-EGF antibody titers increased as a result of vaccination. These findings were then correlated with decreased levels of serum EGF and patient survival. A phase II trial included 80 patients with NSCLC (stage IIIB or IV) after first-line chemotherapy and demonstrated a decrease in EGF concentration in patient serum. A strong correlation was found between antibody titer and reduction in EGF concentration. Reduction of EGF concentration to below 168 pg/mL is associated with prolongation of overall survival (13 months with 168 pg/mL or less *vs.* 5.6 months above 168 pg/mL). High initial concentration is a predictive factor of vaccine response and an adverse prognostic factor for non-vaccinated patients. A phase III clinical trial is ongoing (85,123).

A phase I trial in stage III–IV NSCLC is investigating vaccines targeting indoleamine-2,3-dioxigenase (IDO), an immune regulatory protein that suppresses activity of $CD8^+$ cytotoxic T cells. To date, long-lasting clinical benefits have been demonstrated in almost half of the patients (124).

GV1001 is a telomerase-based vaccine used in clinical trials in NSCLC patients previously treated with chemotherapy and radiotherapy (85). In a phase II trial (CTN-2006), 23 stage III patients received radiotherapy and docetaxel followed by GV1001 vaccination. Long-term immunomonitoring showed durable responses in 13 patients. Immune responders achieved a median of 371 days survival, compared with 182 days for non-responders. In another clinical trial (CTN-2000), 26 patients were vaccinated with two telomerase peptides (GV1001 and I540). Thirteen developed a GV1001 response and achieved increased survival compared with non-responders (median survival 19 *vs.* 3.5 months, respectively) (125).

The Wilms tumor antigen-1 (WT-1) is found in most NSCLC and SCLC patients (126) and a clinical trial tested a 9-mer of WT-1 in several tumor types. Three of 10 lung cancer patients showed an immunological response and one patient continues to survive following repeated vaccinations over more than 2 years (127). WT2725 is a peptide vaccine derived from Wilms tumor protein; a clinical trial in SCLC is also ongoing (85).

Cyclophilin B is found in lung cancer patients and can be a target of CTLS (128). A cyclophilin-based vaccine is being tested in a phase I trial, though no significant increases in cellular response have been observed.

TGF-β2 is released by tumor cells in their microenvironment to protect themselves from immune system. Expression of TGF-β2 has been correlated with poor prognosis in NSCLC (129). Lucanix (Belagenpumatucel-L) is a vaccine consisting of allogeneic NSCLC cell lines transfected with an antisense plasmid to TGF-α2, designed to block TGF-β secretion. A phase II clinical trial in 75 NSCLC patients (stages II–IV) has been completed. The estimated probability of surviving 1 or 2 years was 39% and 20% for patients receiving a low dose of the vaccine and 68% *vs.* 52% for the higher doses. Estimated median survival time for patients on the low dose was 252 *vs.*

581 days for the high dose (129). This vaccine in now in a phase III study (STOP) with 532 patients enrolled. This trial did not meet its primary endpoint, with median overall survival of 20.3 months in vaccine-treated patients treated *vs*. 17.8 months in the group control, but a marked improvement in survival has been detected in specific subgroups of patients (85,113).

The IDM-2101 peptide vaccine is based upon ten different HLA-A2 restricted epitopes against five different antigens (CEA, p53, HER2, MAGE-2 and MAGE-3 antigens along with a pan-DR epitope). A phase II study has been completed and demonstrated immune response (130).

DC vaccines: most smoking-related cancers have p53 mutations and DC vaccines are based on infecting DCs with p53 adenoviruses (131). In *in vitro* experiments, when these transfected DCs are activated they can generate CTLs against p53 (132). In SCLC patients, a significant immune response is induced and patients are sensitized to chemotherapy (133). Cyclophosphamide followed by vaccinations with tumor-antigen-loaded, DC-derived exosomes inhibits Treg functions, restoring T and NK cell effector functions and activating cell immunity. This is currently being studied in phase I trials (85).

Conclusions

The study of two different scientific fields such as stem cell research and cancer immunology and the links between the two could be crucial to develop new therapeutic approaches to prevent metastasis and development of therapy resistance. CSCs/TICs are characterized by low immunogenicity and immunosuppressive activity. They defend themselves from the immune system and adapt to modifications in the tumor microenvironment caused by chemotherapy or radiotherapy. After chemotherapy and radiotherapy, some resistant cells remain that could be detected and partially killed by the immune system. Equilibrium subsequently occurs between immunological elimination and growth of cancer cells and during this period cells may suffer some changes, giving rise to a poorly immunogenic stem cell subpopulation that is not recognized by the immune system. The molecular identification of immunomodulating agents that can reverse or inhibit CSC/TIC escape from immunosurveillance should allow design of new immunotherapy protocols targeting CSCs/TICs. Immune checkpoint blockade has shown promising results in clinical trials in lung cancer. Responses tend to be durable, but there are problems with inter-patient heterogeneity of responses and appropriate patient subpopulations need to be identified. The tumor microenvironment could play a major role in modulating immune response. The success of immunotherapeutic approaches will depend on a better understanding of the basic biology of immune responses and, in particular, the role that tumor microenvironment plays in shaping immune responses. Vaccines targeting stem cells genes, however, are not without potential risks and adverse effects. The most obvious risks relate to pathways shared with normal stem cells. Research into combination of CSC/TIC-targeting antibodies and/or vaccines with conventional cancer therapies at the optimum moment during the course of the disease, and the identification of suitable biomarkers could improve cancer treatment, is therefore crucial.

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Footnote

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Release brakes: chimeric antigen receptor T cells with PD-1 switch receptor

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Adoptive cell therapy chimeric antigen receptor (CAR) redirected T cells is efficacious in the therapy of hematologic malignancies, however, challenging in the treatment of solid cancer due to massive immune repression within the tumor lesion. Recent reports by Kobold *et al.* (1) and Liu *et al.* (2) explored the concept to provide CD28 costimulation by a co-expressed "switch receptor" targeting PD-1 ligands to overcome repression and to make the redirected T cell response more durable in the tumor tissue.

Adoptive cell therapy with CAR redirected T cells showed impressive efficacy in the treatment of hematologic malignancies, strongly suggesting that specific T cells can control sustainable tumor regression (3,4). However, the same efficacy was not observed in the treatment of solid tumors. A major cause is thought to be the inhibitory environment which directly or indirectly prevents a productive and lasting T cell anti-tumor response (5). Multiple inhibitory mechanisms were so far identified involving cells with inhibitory receptors, immune checkpoint inhibitors or secreted factors or metabolic products among others (6). In this context, the programmed cell death protein-1 (PD-1, CD279) is one of the central inhibitory receptors expressed by activated T cells; the corresponding ligands PD-L1 and PD-L2 are expressed by cancer and stroma cells of a variety of tumor entities. While a physiologic role of PD-1 is to limit the T cell activity in the periphery, to terminate an acute inflammatory response and to prevent auto-immunity, many types of cancer share the same mechanism by expressing PD-1 ligands. When engaged by one of its ligands, PD-1 activates the phosphatase SHP2 (7) which inhibits several kinases involved in T cell activation, thereby protecting the tumor tissue against a productive T cell attack. This situation provides a strong rationale to revert the inhibition through administration of blocking antibodies specific for the PD-1 receptor or its ligand PD-L1 (8). Such antibody-mediated immune checkpoint inhibition improved anti-tumor immunity in a number of cases and entered clinical practice in a variety of tumor entities; the presence of T lymphocytes within the tumor tissue is a favorite prognostic marker (9) moreover underlining the central role of a productive T cell response in order to control cancer.

To address the situation for CAR redirected T cell therapy, Liu and colleagues (2) equipped CAR T cells with a so-called switch receptor, PD-1:CD28, which consists of the PD-1 extracellular domain and the CD28 intracellular domain providing costimulation (*Figure 1*). Thereby, the switch receptor targets PD-1 ligands and signals through CD28 without the primary TCR signal to overcome PD-1:PD-L1 mediated T cell suppression. In the used experimental model, T cells were redirected by the 4-1BB-CD3ζ signaling "second generation" CAR towards the clinically relevant tumor antigens mesothelin (MSLN) and prostate-specific cancer antigen (PSCA), respectively. Such

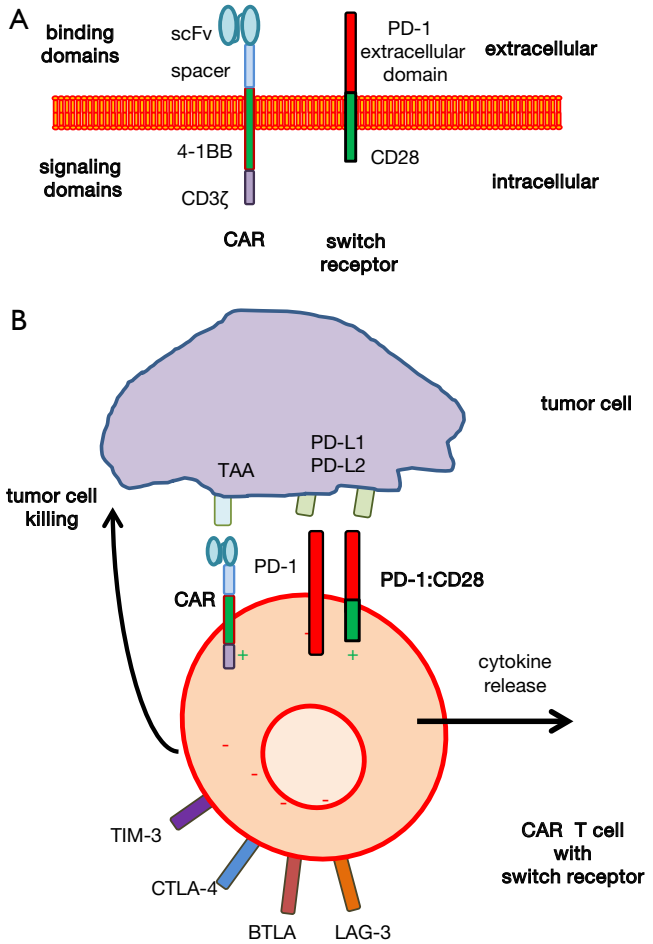

Figure 1 The PD-1:CD28 switch receptor provides CD28 costimulation to overcome PD-1 mediated T cell repression. (A) Schematic diagram depicting the modular composition of the PD-1: CD28 switch receptor and of the "second generation" chimeric antigen receptor (CAR) with 4-1BB and CD3ζ signaling domains. The CAR targets a tumor associated antigen (TAA), the switch receptor binds to the PD-1 ligands PD-L1 or PD-L2 on the cancer cell; (B) CAR signaling upon engagement of cognate antigen on cancer cells does not result in productive T cell activation as long as PD-1 suppression by binding to the respective ligands occurs. The PD-1:CD28 switch receptor provides CD28 costimulation upon PD-1 ligand binding and thereby overcomes PD-1 suppression and improves CAR T cell activation. However, other suppressive mechanisms through TIM-3, CTLA-4, BTLA, or LAG-3 are still in place.

CAR T cells were co-engineered with the switch receptor which provides CD28 costimulation upon engaging PD-1 ligands. Thereby, tumor targeting by CAR redirected T cells is combined with immune checkpoint interference at the cellular level to prevent anergy of CAR T cells in the tumor tissue with high level PD-1 ligand expression.

The PD-1:CD28 switch receptor was initially introduced by Prosser and colleagues (10) who demonstrated that upon PD-L1 binding switch receptor engineered T cells increase ERK phosphorylation, release inflammatory cytokines like IL-2, IFN-γ and TNF-α, increase their proliferative capacity, and enhance the expression of the cytolytic molecule granzyme B. Obviously, PD-1:CD28 switch receptor mediated CD28 signaling acts in a dominant manner over the endogenous PD-1 suppressive signals; moreover, the switch receptor competes for available PD-1 ligands on tumor cells. Such engineered switch receptor T cells show improved anti-melanoma activity in the athymic nude-Foxn1nu mouse model (11).

In accordance to this report, Liu *et al.* (2) observed improved activity of CAR redirected T cells with the PD-1:CD28 switch receptor on large, established, solid tumors in the NOD/scid/IL2rγ$^{-/-}$ (NSG) mouse model. Notably, there is a faster onset of tumor regression and a greater long-term survival rate in those mice treated with T cells with both the CAR and PD-1:CD28 switch receptor compared with T cells with the CAR only. The authors assume that the effect is due higher numbers of specific T cells in the peripheral blood of treated mice. Accordingly, IL-2 increased to 10- to 30-fold in sera of mice treated with switch receptor CAR T cells compared with CAR T cells. The increase of serum IL-2 is assumed to be due to the persistent activation of CAR/switch receptor T cells in the tumor tissue and, on the other hand, to support survival and amplification of the activated T cells. The ability to secrete pro-inflammatory cytokines and to execute cytotoxicity towards tumor cells was retained as revealed by re-administration of those circulating T cells to *in vitro* assays.

The authors hypothesize that the switch-receptor exerts its effect in the long-term through CD28 signaling after PD-L1 binding since a mutated, signal-deficient version of the switch receptor provided no benefit to CAR T cells *in vivo*; such T cells were as efficient as CAR T cells without switch receptor. The conclusion is further underlined by the fact that administration of pembrolizumab, a blocking anti-PD-1 antibody, was less efficient in supporting CAR T cell anti-tumor activity compared to T cells co-grafted with CAR and PD-1:CD28 switch receptor. Consequently, CD28 signaling plays a decisive part in strengthening the anti-tumor effect;

whether other costimulatory domains in a switch receptor provide a similar effect remains to be resolved.

Perspectives

In adoptive cell therapy, the expression of the PD-1:CD28 switch receptor by engineered T cells may have an advantage in providing a costimulatory signal which is required to prevent anergy in the suppressive environment and which is not delivered by the CAR. The same switch receptor may be used to provide additional signals which furthermore support the survival of the redirected T cells. For instance, $CCR7^-$ effector memory cells, which persist in the periphery and are strongly executing cytolysis, are highly prone to activation induced cell death; apoptosis can be prevented by both CD28 and OX40 signals as provided by a "third generation" CAR (12). A switch receptor with OX40 or CD28-OX40 would provide the additional signals in the tumor tissue in order to avoid anergy and apoptosis of the CAR T cells.

The checkpoint ligands PD-L1 or PD-L2 are upregulated by cancer cells in response to the T cell anti-tumor activity which results in a furthermore increase in signaling by the switch receptor (2). The situation may be further exploited by a switch receptor which increasingly provides beneficial agonistic signal and which would increasingly sustain the CAR T cell attack with increasing levels of the suppressive ligand.

The CAR-independent co-signaling by the switch receptor could also be used to complement signaling in order to provide full T cell activation only when the switch receptor delivers costimulation to the primary CD3ζ signal of the CAR. Upon simultaneous engagement of the cancer cell antigen by the CD3ζ CAR and of the PD-1 ligand by the PD-1:CD28 switch receptor, cosignaling would initiate full T cell activation to execute the anti-tumor attack which would not be the case when engaging one ligand only. Complementing signals by co-engagement of two targets likely improves selectivity and safety of the redirected T cell attack.

In this context, preclinical research using immune competent mouse models are required to study the complex interactions between switch receptor engineered CAR T cells and the tumor tissue with a fully established suppressive environment. The model also needs to include the interaction between the transferred T cells with other resident activating or suppressing immune cells in the tumor tissue. Most studies so far use immune deficient models lacking mature T cells, B cells, and natural killer cells, having reduced dendritic cell and macrophage activity and deficiency in the hemolytic complement system; the value to explore efficacy and safety in clinical application is limited.

Despite improved anti-tumor activity of CAR T cells with the PD-1:CD28 switch receptor, the cause of T cell hypo-function at the tumor site remains multi-factorial and alternative pathways are still in place (*Figure 1*). For instance, T cell intrinsic inhibitory enzymes such as SHP-1 and SHP-2 and surface inhibitory receptors like lymphocyte-activated gene-3 (LAG-3) or T cell immunoglobulin mucin-3 (TIM-3) are also upregulated upon T cell activation (13,14). Antibody mediated PD-1 blockade is frequently counteracted by the upregulation of the alternative checkpoint TIM-3 (15) which occurs together with the increase in LAG-3 expression in CAR T cells upon engagement of target (2). Accordingly, CAR T cells with the PD-1:CD28 switch receptor showed reduced expression of both LAG-3 and TIM-3/CEACAM1.

Some suppressor receptors are not only displayed by the cancer cells but also by the tumor stroma or tumor infiltrating suppressor cells. For instance, myeloid derived suppressor cells (MDSCs) in the solid tumor lesion express high levels of PD-L1. Also in this situation antigen-specific T cells with a switch receptor executed an improved anti-tumor response; Kobold and colleagues observed an increased accumulation of IFN-γ producing T cells and an increased ration of $CD8^+$ T cells to MDSCs in the tumor tissue (15). Moreover, mice rejecting the tumor were protected upon subsequent challenge with antigen-positive tumors indicating the establishment of a memory response by such T cells.

All these strategies still remain directed against one suppressive mechanism while multiple other and alternative ways of T cell repression are in place. Future research needs to address the complexity of the tumor situation, e.g., by targeting the key suppressors in a concerted action or by targeting the key regulator in the suppressor signaling pathway.

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Footnote

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PD-L1 copy number gains: a predictive biomarker for PD-1/PD-L1 blockade therapy?

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Immune checkpoint blockade has emerged as a promising and distinct treatment strategy and has caused a paradigm shift in oncology. Significant response and survival benefit have been observed in a fraction of patients who were treated with immune checkpoint inhibitors (ICIs) in several malignancies including melanoma (1-3), non-small-cell lung cancer (NSCLC) (4-7), gastric cancer (8), urothelial carcinoma (9), Hodgkin's lymphoma (10), and head and neck squamous cell carcinoma (11). Additionally, because clinical response to ICI treatment has been seen to vary from to patient, a predictive marker that provides insight on patient response is urgently needed. Currently established companion diagnostics include the HercepTest™ immunohistochemistry (IHC) staining for breast and gastric cancers, fluorescence in situ hybridization (FISH) assays to disclose anaplastic lymphoma kinase (*ALK*) translocations for NSCLC, and mutation analyses for the epidermal growth factor receptor (*EGFR*) gene in NSCLC and *BRAF* for melanoma. Individual differences in treatment efficacy of ICIs may be due to the complex interaction of the tumor microenvironment; where tumor, immune, and stromal cells closely interact. Thus, it has been difficult to establish simple determinants that would predict the efficacy of ICIs like those currently used in targeted therapy.

Immune checkpoints are regulated by many signaling processes that are in part controlled by key players such as programmed death 1 (PD-1), programmed death ligand-1 (PD-L1/CD274), programmed death ligand-2 (PD-L2/CD273), cytotoxic T lymphocyte antigen-4 (CTLA-4), lymphocyte activation gene 3 (LAG-3), and T cell immunoglobulin and mucin domain 3 (TIM-3). Among these interactions, blockade of the PD-1 or its corresponding ligand, PD-L1, has been thoroughly investigated.

The mechanism by which T cells survey and assess cellular antigens depends, in part, on the interaction of inhibitory and activating domains that maintain the immunological synapse. Tumor-specific neopeptides that are created by some of tumor nonsynonymous mutations are recognizable by T cells (12). Thus, ongoing investigations aim to utilize these neopeptides and allow for their recognition and downstream T cell activation using anti-PD-1/PD-L1 immunotherapy. In the case of the PD-1 pathway, activated T cells express PD-1 on their surface and may result in exhausted or inactivated phenotype when PD-1 engages either PD-L1 or PD-L2 (13). PD-L1 is expressed by a variety of cells including those in the tumor microenvironment such as antigen-presenting cells, endothelial cells, and tumor cells. Two anti-PD-1 antibodies, nivolumab and pembrolizumab, and a PD-L1 inhibitor, atezolizumab, were recently approved by the US Food and Drug Administration (FDA), and have recently been applied to patients with a variety of advanced cancers.

Several predictive biomarkers for PD-1/PD-L1 axis

blockade have been suggested. The overexpression of PD-L1 protein on tumor cell surface and/or tumor-infiltrating immune cells (TIICs) was associated with better response to the therapy (2,4,7,9). A PD-L1 IHC test using the clone 22C3 antibody was approved by the FDA as a companion diagnostic for selecting patients with NSCLC for pembrolizumab while another PD-L1 IHC test using the clone 28-8 antibody was approved as a complementary assay for nivolumab in NSCLC. Clone SP142 has also been approved for the detection of PD-L1 on TIICs for locally advanced or metastatic urothelial carcinoma. However, other studies revealed that the predictive value of PD-L1 expression for the use of PD-1 inhibitors were low (3,5), implying that a proportion of PD-L1-negative patients could have responded and benefited from PD-1/PD-L1-directed immunotherapy. Furthermore, evaluating PD-L1 expression levels using IHC analysis poses several issues that may affect treatment planning. First, PD-L1 protein expression is heterogeneous both spatially (14) and temporally (15). Furthermore, tumor cells, endothelial cells, and TIICs may stain for PD-L1 within the tumor microenvironment. Thus, these context-dependent results of PD-L1 expression, especially when examined among small biopsy specimens, may be skewed and not represent true PD-L1 expression status. Second, standardization of PD-L1 staining and tumor tissue preparation has not been established. Finally, which antibody clone and cutoff point for IHC evaluation should be used has yet to be determined. Although performing and assessing PD-L1 IHC is relatively simple, the aforementioned problems in heterogeneity, reproducibility, and standardization has made it more difficult to compare data and should thus be addressed for the future.

Another candidate predictive biomarker for the blockade of the PD-1 axis is mutation burden of tumor cells. Although it has been well established that the likely cause of melanoma and NSCLC are mutations that arise from ultraviolet radiation and tobacco smoking, respectively, its relevance to anti-PD-1/PD-L1 immunotherapy response was unknown. Recently, higher somatic nonsynonymous mutation burden was reported to be associated with greater efficacy and clinical benefit of pembrolizumab monotherapy (16). Furthermore, mismatch-repair deficiency determined by the microsatellite instability PCR analysis has been shown to predict the treatment benefit of pembrolizumab in a cohort that mainly consisted of patients with colorectal cancer (17).

An additional probable predictor of response to PD-1/PD-L1 inhibitors is the presence of tumor-infiltrating antigen-specific CD8-positive T cells. Recently, cancers have been proposed to be stratified into four different tumor microenvironments based on the presence of TIICs and tumor PD-L1 expression status (18). PD-L1-positive tumors that contain TIICs are classified as a type I tumor microenvironment. This environment is characterized by tumor immunogenicity and is most likely to respond to checkpoint blockade. In melanoma patients with pre-existing tumor-associated CD8-positive T cells, pembrolizumab therapy was shown to inhibit PD-1/PD-L1 mediated adaptive immune resistance and conferred tumor regression (19). It should be noted that oncogene-driven PD-L1 expression, which is diffuse and constitutive, is distinct from adaptive inflammation-driven PD-L1 expression. Although *EGFR* activating mutations (20) and *ALK* translocations (21) in NSCLC were shown to increase PD-L1 expression, both of these gene alterations have been reported to be associated with low response to PD-1/PD-L1 inhibitors, with objective response rates of 3.6% in *EGFR*-mutant or ALK-positive patients versus 23.3% in *EGFR* wild-type and ALK-negative/unknown patients (15). The decreased presence of the type I tumor microenvironment was suggested to be responsible for the low treatment benefit seen among tumors harboring these gene alterations. Because *EGFR*-mutant or *ALK*-rearranged NSCLCs are more common among never- or light-smokers and have less nonsynonymous mutations than smoking-related tumors (22), the immunogenicity of the tumors harboring the *EGFR* or *ALK* gene alterations are relatively low, resulting in less tumor recognition by immune cells.

In a recent issue of *Genes Chromosomes & Cancer*, Budczies and colleagues (23) reported the landscape of *PD-L1* copy number alterations (CNAs) in 22 major cancer types using The Cancer Genome Atlas (TCGA) RNAseq and CNA datasets. They found a strong correlation between *PD-L1* CNAs and mRNA expression levels for most cancers. Notably, they observed that the mutation load was significantly higher in tumors with *PD-L1* copy number gains than in tumors with normal *PD-L1* copy number among eight individual cancer cohorts including the lung adenocarcinoma and lung squamous cell carcinoma cohorts. Interestingly, higher mutation load was also found among *PD-L1* deleted tumors when compared to *PD-L1* normal tumors in seven out of the 22 cancer types. Whether immunotherapy using checkpoint inhibitors is effective in *PD-L1* deleted tumors with a high mutation burden seems to be a topic that requires further investigation.

As written in the article by Budczies and colleagues (23),

CNAs of the *PD-L1* gene have received surprisingly little attention until now. We previously reported the prevalence, clinicopathological characteristics, and prognostic implications of *PD-L1* copy number gains in NSCLC using FISH (24). *PD-L1* amplification and polysomy were observed in 3.1% and 13.2% of patients, respectively, and were independently associated with PD-L1 protein overexpression. *PD-L1* copy number gains were more commonly observed among smoking-related tumors, and strikingly, *PD-L1* gene amplification was found to be exclusive to *EGFR* mutations and ALK expression, both of which were reported to be negatively associated with response to PD-1/PD-L1 inhibitors (15). Furthermore, our data indicated that tumor *PD-L1* copy number status was more consistent and reproducible than tumor PD-L1 protein expression detected by IHC when primary tumors and synchronous regional lymph node metastases were comparatively analyzed.

Similar to Budczies and colleagues (23), Ock and colleagues (25) carried out comprehensive analyses of immunogenomic properties in TCGA datasets and evaluated the RNA expression levels of *PD-L1* and *CD8A*. They observed that the type I tumor microenvironment defined by high *PD-L1* and *CD8A* expression were especially common among lung adenocarcinomas (67.1%) and lung squamous cell carcinomas (63.5%) as well as cancers derived from lymphoproliferative tissues and kidney clear cell carcinoma. Importantly, a high mutation burden and *PD-L1* amplification were independently associated with the type I tumor microenvironment in a multivariate analysis. Taken together, *PD-L1* copy number gains, in particular *PD-L1* amplification, appear to represent the genomic instability of tumor cells in several cancer types. Although *PD-L1* copy number gains, which lead to diffuse and constitutive PD-L1 expression, are one of many mechanisms of innate immune resistance, they also reflect the type I tumor microenvironment with high mutation load in tumor cells.

The search for a biomarker that could accurately predict ICI response has been widely and enthusiastically performed so that rational and full use of the promising and costly therapy can be achieved. Given the complexity of the dynamic interaction of the immune system and tumors, predicting the response of ICI treatment using a single biomarker might not be possible. PD-L1 protein expression has relatively limited power to predict response to PD-1/PD-L1 inhibitors. At present, the wide use of next-generation sequencing data in the clinical setting for evaluating the mutation burden of each patient is not possible. Additionally, it is difficult to precisely predict true neo-epitopes which can be recognized as non-self by T cells from total nonsynonymous mutations tailored to each patient's tumor. As for evaluation of TIICs, inaccurate results might be reported because testing is carried out on a small tissue specimen in a proportion of patients with advanced cancers such as NSCLC. However, evaluating *PD-L1* copy number gains can be relatively simple using FISH even on small biopsy specimens. Moreover, *PD-L1* copy number screening has been suggested to be helpful in assessing accurate PD-L1 protein expression, mutation burden, and specific tumor microenvironments. Thus, conclusively, we believe that the predictive significance of therapy response should be prospectively assessed in clinical trials. We hope to further evaluate the predictive value of *PD-L1* copy number gains in our upcoming clinical trial of patients with advanced NSCLC using PD-1/PD-L1 ICIs.

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Footnote

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Cell intrinsic PD-1 checkpoint blockade releases the brake on human chimeric antigen receptor (CAR) T cells for solid tumors

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The immune system plays an important role in controlling and eliminating cancer. Nevertheless, tumors often evade endogenous immune responses due to tolerogenic mechanisms which prevent the rejection of malignant cells that are recognized as 'self' or alternatively, not 'dangerous.' The complex network of biological pathways that maintain tolerance involves several mediators, including myeloid and lymphoid-derived regulatory cells, immunosuppressive cytokines and chemokines, as well as immune checkpoint molecules that down-modulate anti-tumor immunity. The programmed cell death protein 1 (PD-1)—PD-1 ligand 1 (PD-L1) receptor-ligand pair is a principal immune checkpoint axis operative in the tumor microenvironment that attenuates T cell receptor (TCR)-mediated activation, leading to inhibition of T cell expansion and cytokine production (1-3). Although the genetic re-direction of T cells with chimeric antigen receptors (CARs) is an attractive approach to break tolerance in the setting of cancer, there is a paucity of experimental evidence to suggest that CAR T cells are optimally functional within solid tumors and additionally, that blockade of immune checkpoints can potentiate their activity. Cherkassky *et al*. (4) address this long-standing question by engineering mesothelin-targeted CAR T cells to be intrinsically resistant to PD-1-induced inhibition. These findings should spur future investigations focused on more carefully defining the determinants of CAR T cell exhaustion and assessing the therapeutic potential of individual or combined checkpoint blockade in augmenting anti-tumor T cell responses.

In the case of most human cancers, the TCR repertoire is either lacking or inadequate for tumor control (5-7), which has paved the way for the concept of using CAR-redirected T cells for adoptive immunotherapy. Currently, we and others have demonstrated that CAR T cells can eradicate leukemia and induce long-term durable remissions in patients (8-10). In the setting of solid tumors, however, this approach has not achieved the same degree of clinical success, and this may be attributed to unique barriers imposed by solid tumors that are absent in hematological malignancies. Following the trafficking of CAR T cells to tumor tissues and infiltration through stromal elements, these engineered lymphocytes must overcome challenges present within a profoundly immunosuppressive landscape which, in addition to the aforementioned tolerogenic mechanisms, may include nutrient deprivation, oxidative stress, low pH and hypoxia [reviewed in (11)]. Furthermore, in this microenvironment where CAR T cells must function, there is evidence that TCR signaling becomes uncoupled independently of the effects of tumors on TCRζ expression (12,13). This led us and other groups to construct "bipartite receptors" comprised of TCRζ and intracellular signaling modules such as CD28, 4-1BB, OX40, ICOS or CD27 (14-19) to substantially improve the function and proliferation of adoptively transferred T cells. While the induction of

costimulatory pathways can overcome some degree of solid tumor-mediated inhibitory signaling, the extent to which T cells expressing these "second generation" CARs could be inhibited upon *in vivo* antigen exposure within the solid tumor microenvironment is not well-characterized.

The studies by Cherkassky *et al.* (4) highlight the effects of specific types of co-stimulatory signaling on CAR T cell potency, which have been largely unappreciated due the use of immune sensitive model systems and the infusion of large numbers of T cells that do not accurately reflect effector to target cell ratios attained in patients with high tumor burdens. Similar to previous studies using different *in vivo* experimental systems, T cells expressing anti-mesothelin chimeric receptors bearing TCRζ signaling modules alone were not sufficient to drive sustained antitumor activity in an orthotopic model of malignant pleural mesothelioma (4,14). In contrast, the administration of high doses of CAR T cells incorporating CD28 or 4-1BB endodomains resulted in tumor clearance, regardless of the costimulatory pathway induced. Interestingly, upon transfer of low doses of T cells, only 4-1BBζ CAR T cells resulted in long-term tumor eradication, despite an intratumoral accumulation and persistence comparable to that of their CD28ζ counterparts. These findings were supported by the superior ability of 4-1BBζ CAR T cells to retain effector cytokine production and cytotoxic capacity following *in vivo* antigen exposure (4). In addition, when recall response was assessed (the so-called ability to function as "serial killers"), 4-1BBζ CAR T cells were more resistant to ongoing immune inhibition induced by repeated antigen stimulation *in vitro* or tumor re-challenge *in vivo* (4). These findings underscore that when selecting an optimal costimulatory signaling strategy, mere assessment of CAR T cell engraftment particularly as measured through peripheral blood sampling may not be a sufficient indication of potency in the setting of solid tumor indications. Pre-clinical studies that will ultimately inform clinical trial designs should thus move toward the evaluation of functional correlates of persistence in model systems that more accurately recapitulate tumor antigen load in patients.

Despite the improved anti-tumor activities of second generation CAR T cells in which enhanced persistence is mainly attributed to the engagement of costimulatory pathways, these engineered lymphocytes also undergo an activation-induced upregulation of co-inhibitory receptors and pathways to naturally control the magnitude of immune responses. Accordingly, the balance between positive and negative signals is a critical determinant of the outcome of T cell-mediated immunity. However, the increased expression of inhibitory receptors following antigen encounter coupled with the overexpression of their cognate ligands by cancer cells greatly limits overall anti-tumor activity. Although blockade of negative regulatory checkpoint pathways has recently demonstrated promise in restoring defective T cell function (20-22), the clinical success of these approaches often relies on tumor mutation burden (23) and the localization of T cells within the microenvironment. Further, unabridged immune activation following checkpoint blockade is often accompanied by a number of toxicities and autoimmune sequelae (24). In patients with tumors of insufficient immunogenicity (i.e., "non-inflamed" tumors), treatment with CAR-redirected T cells may address these limitations. Nevertheless, adoptively-transferred T cells are susceptible to immune inhibition, and therefore, the removal of inhibitory checkpoint signals may potentiate their full anti-tumor effects.

Cherkassky and colleagues (4) focused on repairing the defect observed in tumor-infiltrating CD28ζ-signaling CAR T cells that was largely characterized by PD-1 overexpression. Signaling through PD-1 results in the recruitment of phosphatases SHP-2 and to a lesser extent SHP-1 to the inhibitory receptor cytoplasmic domain that initiates dephosphorylation of antigen receptor proximal signaling molecules including ZAP70, PKCθ, and CD3ζ (1), leading presumably to attenuation of the CAR/CD28ζ signal. Co-expression of a PD-1 with a truncated intracellular signaling domain counteracted the inhibitory signaling conferred by endogenous PD-1 (4), which was likely attributed to competition for PD-L1 by the dominant negative receptor (*Figure 1*). This strategy resulted in enhanced *in vitro* CD28ζ CAR T cell activity and significantly improved *in vivo* anti-tumor efficacy with a single administration (4). The strength of this approach is elimination of the requirement for repeated anti-PD-1/PD-L1 antibody dosing, which the authors also demonstrate to be efficacious when administered concurrently with CD28ζ CAR T cells. Indeed, this genetic engineering strategy may offer an enhanced safety profile as well as a therapeutic benefit over PD-1/PD-L1-targeting antibodies due to pathway inhibition that is restricted to adoptively-transferred T cells. This permits tumor-targeted T cell reinvigoration within the microenvironment without dose-limiting toxicities that are inherent to broadly applied antibody-based checkpoint blockade (24). In future investigations focused on extending this approach into clinical development, consideration should be given to the

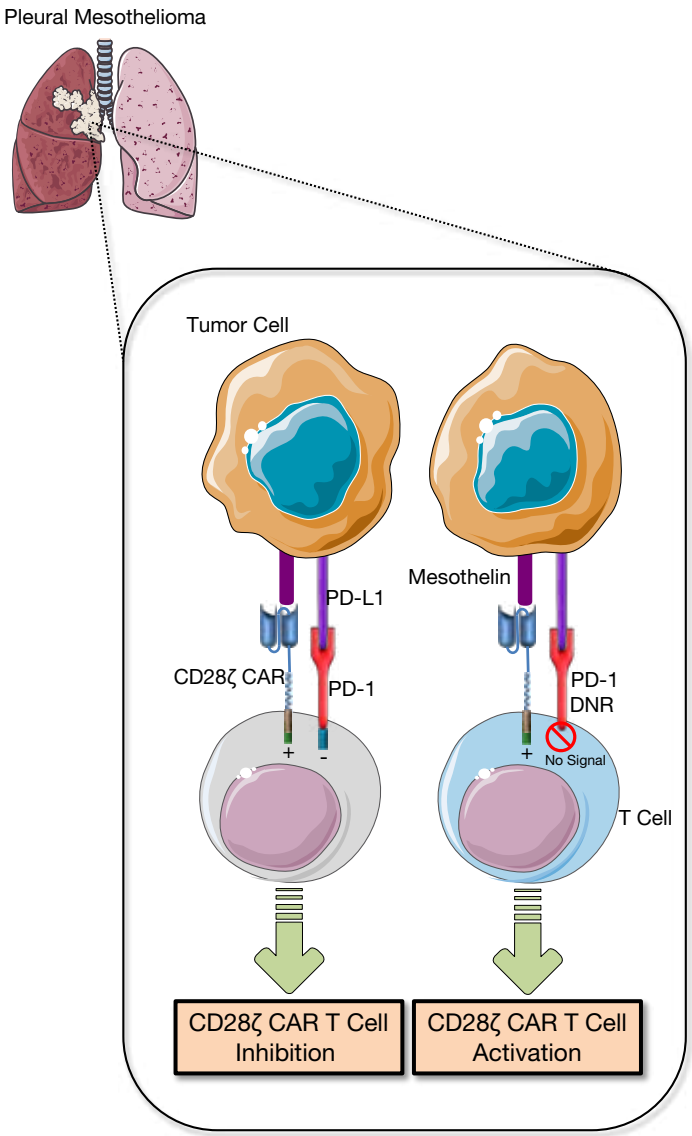

Figure 1 Inhibition of PD-1 signaling in CD28ζ CAR T cells potentiates anti-tumor function in a model of metastatic pleural mesothelioma. Schematic representation of CD28ζ CAR T cells associating with their cognate antigen (mesothelin) and also binding to PD-L1 via endogenous PD-1 (co-inhibitory signal) leads to functional inhibition of these redirected T lymphocytes (left). Abrogation of PD-1/PD-L1 signaling by co-expression of a PD-1 dominant negative receptor (DNR) with CAR renders T cells resistant to immune inhibition and potentiates activation and resultant anti-tumor activity (right). PD-1, programmed cell death protein 1; PD-L1, PD-1 ligand 1; CAR, chimeric antigen receptor.

incorporation of inducible suicide genes into CAR T cells that co-express a dominant negative PD-1 receptor. This "safety switch"-type engineering may ultimately prevent or limit the potential for adverse lymphoproliferative or autoimmunity.

How might these findings improve treatment strategies based on adoptive T cell transfer? Cherkassky *et al.* (4) illustrated the functional significance of a central mechanism underlying T cell exhaustion in an antigen-dependent model system that recapitulates certain features of inhibitory signaling in the tumor microenvironment. In addition, the authors demonstrate the importance of selecting an optimal

costimulatory strategy that is capable of withstanding immune inhibition. It is intriguing to speculate that CAR T cells containing tripartite signaling domains may be more resistant to immune inhibitory effects imposed by this model, due to their "hardwired" costimulatory signals that are triggered upon encounter with tumor antigen. There are theoretical reasons to suggest that three signaling modules comprising a "third generation" chimeric receptor might have additive or synergistic effects. Although 4-1BB can function independently of CD28 (25), it is also possible that one of these co-stimulatory signals will be dominant. Nevertheless, deep immunophenotyping in addition to relevant functional analyses of pre- and post-infusion CAR T cells may provide clues about which T cells can proliferate and elicit superior anti-tumor activity following inhibition of the PD-1 inhibitory pathway. For example, it has been recently demonstrated that a certain PD-1+ CD8+ T cell population that proliferates following blockade of PD-1/PD-L1 signaling expresses several costimulatory molecules (e.g., ICOS, CD28) and is capable of self-renewal as well as effector differentiation (26). Thus, selection of such a population of T cells for CAR-based strategies that combine checkpoint blockade may optimize PD-1-directed immunotherapy. Finally, it is becoming increasingly evident that T cell exhaustion is regulated by co-expression of multiple inhibitory receptors including PD-1 (27). A systematic exploration of all inhibitory pathways that could limit CAR T cell potency in the setting of the solid tumor microenvironment may elucidate the therapeutic potential of individual or combined blockade of additional inhibitory receptors in restoring anti-tumor T cell function.

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Footnote

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Evoking durable anti-cancer responses with blocking antibodies to PD-1 and PD-L1

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Inhibitory immune checkpoints play a critical role in regulating the strength and duration of immune responses in order to maintain self-tolerance and prevent autoimmunity (1). Such regulatory mechanisms are typically exploited by tumors as an immune escape mechanism and thereby pose a major obstacle to the induction of clinically relevant anti-cancer immune responses capable of controlling and/or eradicating disease (2). Efforts to safely re-engage endogenous anti-tumor immunity has seen the exciting development of immunotherapeutic antibodies designed to selectively block the interaction of inhibitory receptors with their ligands with the goal of enhancing the anti-tumor activity of T cells. The first antibody of this nature to be successfully trialed in the clinic was ipilimumab (Yervoy, Bristol Myers Squibb, Princeton, NJ) previously known as MDX-010 (Medarex, Princeton, NJ), targeting the immunoregulatory receptor cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) [Reviewed in (3)]. Expressed on activated T cells, CTLA-4 signalling can attenuate T cell function through competing with the co-stimulatory molecule CD28 for its B7-ligands on antigen presenting cells (APC) (4). In 2011 ipilimumab was approved by the US Food and Drug Administration for the treatment of stage IV melanoma and is now being trialed for activity in patients with lung and prostate cancer. Such positive advances of passive cancer immunotherapy into mainstream oncology have been the driving force behind the development of new blocking antibodies to immune checkpoints for cancer therapy and their integration into early phase clinical trials.

The therapeutic promise of antibody mediated blockade of programmed death (PD)-1 for the treatment of cancer has emerged from findings demonstrating that this co-inhibitory receptor, which plays an important role in regulating immune cell exhaustion within peripheral tissue, is commonly expressed on tumor-associated immune infiltrates (3). Tumors are also highly infiltrated by T-regulatory cells that typically express high levels of PD-1, the signalling through which may promote their expansion and/or suppressor activity (5). The most dominant immunosuppressive ligand of PD-1 is Programmed Death-Ligand 1 (PD-L1), which is expressed on both mouse and human tumor cells, and tumor associated stroma and non-transformed immune cells including dendritic cells (DC) (3). Tumor associated expression of PD-L1 has been shown to confer immune resistance and potentially protect tumor cells from T-cell mediated apoptosis (6,7); a phenomenon that can be over-ridden with targeted blocking antibodies to PD-1 or PD-L1 resulting in the induction of enhanced T cell function (2). In cancer patients, PD-L1 expression has been associated with poor outcome (8,9), providing a strong rationale for pursuing the development of inhibitory antibodies to this pathway for cancer immunotherapy.

The much-anticipated findings of two early phase clinical studies trialing the activity and safety of therapeutic antibodies to PD-1 and its ligand PD-L1 in advanced solid cancer were recently published in back-to-back articles in *the New England and Journal of Medicine* (10,11). In the first in-human Phase I study of the fully human IgG4 PD-1 monoclonal antibody in patients with advanced solid tumors (mAb), BMS-936558 (previously known as MDX-1106, BMS, Princeton, NJ), a single-dose regimen was found to be well tolerated and associated with evidence of anti-tumor activity (12). In the recent study by Topalian et al., the anti-tumor activity and safety of this antibody was assessed in

a multi-dose Phase I study involving 296 patients with a diverse range of advanced solid cancer (10). The BMS-936558 antibody was administered at 1-10 mg/kg of body weight every 2 weeks over an 8-week treatment cycle, with up to 12 cycles. Of the 296 patients enrolled, 1 in 4 to 1 in 5 patients with melanoma (28%, 26 of 94 patients), non-small-cell lung cancer (18%, 14 of 76 patients) or renal-cell cancer (27%, 9 of 33 patients) had durable (≥24 weeks), objective (complete or partial) responses (10), as determined by the Response Evaluation Criteria In Solid Tumors (RECIST). Notably, within these patient cohorts, drug-activity was detected in multiple sites of metastases including the liver, lung, lymph nodes and bone. No objective responses were observed in patients with colorectal (19 patients enrolled) or prostate cancer (17 patients enrolled). Given the advanced status of disease in all patients enrolled in this study and the refractive nature of the cancers, the observed activity as well as durability of the responses to BMS-936558, relative to other more conventional therapies, is highly significant.

Interestingly, objective responses were not observed in patient tumors in which tumor-cell expression of PD-L1 was not detected. Of the 42 tumor biopsies taken across all three responding cancer types, 25 were found to be positive for PD-L1 and 36% of these demonstrated an objective response to BMS-936558 therapy (10). Notably, PD-L1 expression is typically up regulated in inflammatory microenvironments in response to proinflammatory cytokines such as IFN-γ and has been suggested to reflect tumor cell adaption to endogenous immune responses (9). Based on this it would be interesting to assess whether PD-L1 expression, particularly in patients in whom objective responses were observed, also correlated with a positive immune score at the initiation of therapy. This preliminary indication that tumors positive for PD-L1 have an increased potential to support an objective response to BMS-936558 drug therapy highlights the potential importance of this immunosuppressive ligand as a predictive biomarker of response; an outcome that may not have been predicted based on preclinical tumor studies in the mouse in which minimal single-agent activity has been reported despite detectably high levels of PD-L1 expression on ex-vivo analysed tumors (13). This disparity may relate to differences in the suppressive barriers that exist within the mouse and human tumor microenvironments and/or the rates with which the disease evolves and/or progresses. Immune suppression associated with prior drug treatments and/or other tumor/host regulatory factors, owing to the advanced nature of the cancers treated in this study, may account for why only a third of the confirmed PD-L1-positive tumors demonstrated an objective response.

The lack of objective responses in all patients with PD-L1-negative tumors raises the question as to whether blocking antibodies to PD-L1 could be a more selective means of disarming this immunosuppressive pathway within tumors. In a companion Phase I study Brahmer JR *et al*., (11) reported on the activity of the BMS-936559 drug, a high affinity fully human PD-L1 specific IgG4 mAb, capable of inhibiting PD-L1 binding to PD-1 as well as CD80 expressed on T cells (and possibly APC); the significance of which is still unclear. In this study, BMS-936559 was administered intravenously at 0.3-10 mg/kg of body weight every 14 days in 6-week cycles for up to 16 cycles. A total of 207 patients with advanced solid cancer were enrolled in the study in which durable (≥24 weeks) objective response rates of 6-17% were induced in a range of different cancer types including melanoma (17%, 9 of 52 patients), renal cell cancer (12%, 2 of 17 patients), non-small cell lung cancer (10%, 5 of 49 patients) and ovarian cancer (6%, 1 of 17 patients) (11). No objective responses have been observed in patients with colorectal (18 patients enrolled) or pancreatic cancer (14 patients enrolled) and no activity of the antibody was evident in patients with gastric (7 patients enrolled) or breast cancer (4 patients enrolled). Notably, the pattern of clinical activity of the BMS-936559 drug was similar to that of the anti-PD-1 mAb used in the Topalian study; however, the frequency of objective responses to the anti-PD-L1 antibody was lower. It will be important to ascertain whether this level of activity correlates with PD-L1 expression on the tumor cells, thus reaffirming the immunosuppressive dominance of tumor cell associated PD-L1.

Collectively the toxicity profiling from both studies would suggest that the PD-1 and PD-L1 targeted antibodies were largely well tolerated. Grade three and four drug associated adverse events, with potential immune-related causes were identified in 14% and 9% of patients, respectively (10,11), however, these appeared to be less severe than that which has been reported for ipilimumab [Reviewed in (14)]. Notably, in the Topalian study 9 of the 296 patients (3%) developed pneumonitis of whom three died due to this drug-related complication. This outcome has raised awareness of the potentially important role that the PD-1/PD-L1 pathway plays in regulating inflammatory responses to pathogenic microbes. Indeed PD-1 deficient mice were reported to have a significantly reduced ability of controlling fatal inflammatory responses in the lung after Mycobacterium tuberculosis infection compared to wildtype mice (15). Notably, M. tuberculosis infected PD-1-/- mice developed severe multifocal necrotic pneumonia. Ultimately, a greater understanding

of the role that PD-1/PD-L1 signaling plays in controlling inflammation in the lungs in response to different types of infections will help to identify those patients who may be more susceptible to this drug-related adverse event as well as better manage the condition in future trials.

The question of how best to integrate the use of PD-1/PD-L1 blocking antibodies into mainstream oncology, for the safe and effective treatment of cancer, is a subject of ongoing investigation. More extensive histological analysis of patient tumors for PD-L1 expression and immunological assessment of the tumor microenvironment and immune infiltrates of pre- and post-therapy patient biopsies will be the key to identifying viable biomarkers that will ensure optimal clinical application of these immunotherapeutic agents. Ultimately however, the true clinical benefit of these immunotherapeutic agents across a broad range of cancer types will likely be best realised when used in combination with select chemotherapeutics, radiotherapy, HER-2 targeted therapies, anti-CTLA-4 or anti-cancer vaccines, all of which have the capacity to stimulate endogenous anti-tumor immunity. By selectively breaking down key immunosuppressive barriers within tumors, like that of PD-1, the full therapeutic power of important first-line and experimental anti-cancer therapies will be unleased. Collectively the findings from the Topalian *et al*., and Brahmer *et al*., studies provide strong validation for pursuing the clinical development of blocking antibodies to PD-1 and PD-L1 as part of our increasing immunotherapeutic armament against cancer.

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Footnote

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Uncovering first molecular mechanisms of secondary resistance against PD-1 blockade

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Provenance: This is a Guest Commentary commissioned by Section Editor Xuewen Zhang, MD (Department of Medical Oncology, Sun Yat-Sen University Cancer Center, Guangzhou, China).

Comment on: Zaretsky JM, Garcia-Diaz A, Shin DS, *et al*. Mutations Associated with Acquired Resistance to PD-1 Blockade in Melanoma. N Engl J Med 2016;375:819-29.

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Checkpoint inhibition by anti-PD-1 antibodies alone or in combination with CTLA-4 blockers is the new paradigm in the treatment of metastatic melanoma (1,2). Response rates are high with about 40% in anti-PD-1 monotherapy and about 60% in combination with anti-CTLA-4 inhibitors (3). Initially, responses were assumed to be durable, but soon it became evident that even deeply responding patients with completely decreased metastases bear the potential to relapse even under continuously ongoing therapy (4,5). The molecular mechanisms of this acquired resistance remained unclear until now, when Zaretsky and coworkers from the group of Antoni Ribas succeeded to unravel some first molecular mechanisms helping to understand this complex clinical situation (6).

The authors performed an elaborate molecular workup of tumor tissues obtained from four melanoma patients treated with the PD-1 inhibitor pembrolizumab. They compared the results obtained by whole exome sequencing of tissue samples taken before the onset of pembrolizumab and at the time of secondary disease progression. Thereby, in three out of four patients genetic alterations in molecular pathways essential for interferon (IFN) signaling and antigen presentation were detected, which appeared under ongoing treatment and thus are likely to represent immune escape mechanisms evolving under the selective pressure of anti-PD-1 therapy.

It has been observed, that tumors without any T cell infiltrate before therapy achieve a dense CD8+ T cell infiltrate under ongoing anti-PD-1 treatment paralleled by a clinical response. Interestingly, in many tumors progressing at a later time point under ongoing therapy, these T cell infiltrates remain present. This observation indicates that the mechanisms attracting the T cells were still present, but their capacity to attack the tumor cells had developed new dysfunctionalities in terms of new immune escape mechanisms. These mechanisms could hypothetically be a dysfunction in the cytotoxic properties of the T cells, an inactivation of the T cells by cytokines and/or checkpoint molecules other than PD-1, a loss of tumor antigens, or a defect of the antigen processing and presentation machinery. The findings of Zaretsky and coworkers reveal the latter mechanism of a defective antigen processing and presentation as most relevant in melanoma with a secondary resistance to anti-PD-1, as far as this can be estimated from the relatively low number of four patients analyzed.

In one of the four patients a frame-shift deletion was found in exon 1 of the beta-2-microglobulin (B2M) gene. B2M has an essential function supporting HLA class I molecules to stabilize their cell surface expression and to enable their capacity to present tumor-specific class I peptides. Loss-of-function mutations in B2M have already been described as a highly relevant strategy of tumor cells to escape from T cell-mediated immune responses by protecting the tumor cells from T cell recognition (7,8). Remarkably, in two of the four patients analyzed, loss-of-function mutations were detected in the genes encoding

for interferon-receptor-associated Janus kinase 1 (JAK1) or Janus kinase 2 (JAK2), resulting in a lack of response to IFN gamma and its signaling cascade. Normally, IFN gamma pathway activation exerts anti-tumoral effects by a reduction of cell proliferation as well as an enhancement of antigen presentation and hereby tumor cell recognition by cell-mediated immune responses. The authors additionally performed functional assays showing that the tumor cells bearing JAK mutations were insensitive to IFN gamma as shown by a lack of phosphorylation of the signal transducer and activator of transcription 1 (STAT1), which is the key molecule in the IFN gamma signaling pathway.

Notably, Zaretsky and coworkers had to screen 78 metastatic melanoma patients treated with pembrolizumab at their institution to find four patients suitable for the intended molecular workup. Patients had to demonstrate an objective response under pembrolizumab therapy, which had to be confirmed four weeks later using RECIST criteria. Moreover, the patients had to show a late relapse under ongoing pembrolizumab after at least six months of documented clinical response. Finally, tumor tissue samples must have been available from before onset of pembrolizumab and from disease progression. This low rate of 5% of suitable patients with evaluable tumor materials although obtained at a university medical center indicates the need to strongly increase the frequency of diagnostic tumor biopsies taken from patients during their course of disease. Particularly in melanoma with its high rate of easily accessible metastases to the skin and lymph nodes, the procedure of sequential biopsy of these lesions should enter the routine clinical practice with the intent to gain molecular insights into the biology of the individual patient's tumor.

Taken together, the study of Zaretsky and coworkers demonstrated for the first time, that an immunotherapy with checkpoint inhibitors is able to induce genetic aberrations, translating into immune escape mechanisms in tumor cells which are thereafter clonally expanded by selective pressure, and hereby lead to a disease relapse in initially responding patients. In the present work only four patients could be analyzed. Thus, it can be assumed that after analysis of further suitable patient materials, in the near future a larger diversity of molecular mechanisms of secondary resistance to PD-1 inhibitors will be discovered.

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Footnote

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Program death-1 (PD-1) receptor pathway inhibition in cancer medicine: a perspective on clinical efficacy and associated toxicities

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Comment on: Eigentler TK, Hassel JC, Berking C, *et al.* Diagnosis, monitoring and management of immune-related adverse drug reactions of anti-PD-1 antibody therapy. Cancer Treat Rev 2016;45:7-18.

Abstract: Enlisting the host's immune system to destroy and eradicate tumors in patients with advanced cancer has long been pursued by researchers and clinicians worldwide. The development of immune checkpoint inhibitors—agents targeting co-stimulatory T cell receptors or their ligands—have demonstrated substantial and durable anti-tumor activity in selected patients with different tumor types, renewed our enthusiasm for immunotherapy, and generated large research efforts, establishing Immuno-Oncology as a solid discipline of cancer medicine. The first immune checkpoint inhibitor, ipilimumab—a cytotoxic T lymphocyte associated antigen-4 (CTLA-4) inhibitor received FDA approval for patients with melanoma in different stages of disease. PD-1 inhibitors have better efficacy and safety profile than their predecessor. Several agents inhibiting this pathway have completed early stages of drug development (biology, pharmacokinetics, safety, efficacy, etc.) and are rapidly finding their way to the clinic. Of these, three already received FDA approval for different indications: Nivolumab (Bristol Myers Squibb), Pembrolizumab (Merck & Co), and Alemtuzumab (Genentech). As expected, their toxicity profile predominantly includes immune related adverse events. The majority of these adverse events are manageable and grade 3/4 toxicities are only observed in 1–3% of patients. Other aspects of clinical interest include: (I) while toxicities are consistent among different agents, their incidence vary slightly among different tumor types; (II) stabilization of tumor growth is observed in a large number of patients; however, objective responses are still reserved to a minority; (III) PD-L1 expression on tumor cells is the most predictive biomarker. Nonetheless, a considerable number of PD-L1(–) patients experience objective responses and differences in survival according to PD-L1 status are not uniform; and (IV) when compared with cytotoxic chemotherapy and other targeted therapies, the duration of responses and safety profile seem to be major advantages among responders to this group of novel biologicals.

Keywords: Cancer immunotherapy; immunological tolerance; program death-1 receptor (PD-1 receptor)

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Cancer immunotherapy

Inducing specific recognition and destruction of tumors by the host's immune system has been a promising but elusive treatment strategy for several decades. Historical observations of striking immune mediated anti-tumor responses among cancer patients have stimulated substantial research efforts aimed at identifying the factors involved in these processes. Clinical observations of patients who

attained substantial tumor responses after episodes of systemic infections caused by Streptococcus pyogenes, led Dr. Coley to design a series of experiments injecting streptococcal cultures to patients with sarcoma to evaluate the potential to induce immune cross-reactivity and eradication of tumor cells (1,2). Subsequent efforts culminated with the approval of intracavitary administration of Bacillus Calmette-Guerin (BCG) to treat patients with superficial non-muscle invasive urothelial carcinomas of ther bladder (3), interferon for several tumor types (4-6), and interleukin-2 for melanoma and renal cell carcinoma (7,8), among others.

Immunological tolerance

Normal individuals are tolerant to their own antigens and discriminate against foreign antigens. During the maturation process in lymphoid organs—usually before birth, all lymphocytes undergo a phase, in which antigen exposure results in tolerance instead of activation. Clones of lymphocytes that become active when exposed to self-antigens are suppressed to avoid responses against self-antigens. This process is known as central tolerance. Alternatively, peripheral tolerance is induced by the recognition of antigens without adequate levels of co-stimulators—which are necessary for the activation of lymphocytes or by the repeated and persistent stimulation by self-antigens in peripheral tissues.

The term immunological tolerance was first described by Sir Frank Burnet in 1949 and later confirmed by Billingham, Brent, and Medawar in 1953 through experiments with different strains of *CBA* and *A* strain mice and Rhode Island Red and White Leghorn chicken (9). In these experiments, the investigators, demonstrated: (I) that immunological tolerance develops in utero. Mice and chicken never or very limitedly develop strong immunological reactions against foreign antigens inoculated in utero. These animals become tolerant to the inoculated tissue and to re-exposure to the same antigen in their adult life; (II) acquired immunological tolerance is highly specific: The inoculated animals maintained their tolerance for the originally exposed antigens, while rejecting other foreign tissues; (III) acquired immunological tolerance is due to a host's specific acceptance of foreign antigens rather than a modification in the inoculated tissues. These pivotal discoveries made Sirs Medawar and Burnet the recipients of the Nobel prize of Medicine in 1960 and served as cornerstone for the development of organ transplantation and modern immunological therapies (10). Moreover, the role of immunological tolerance in the etiology of cancer became widely recognized (11).

"Programmed death-1" (PD-1) receptor and its ligands

PD-1 (CD279) is an Immunoglobulin superfamily member expressed in a subpopulation of CD4– CD8– normal thymocytes and induced in peripheral lymphocytes following activation. Ishida, Honjo and others discovered the receptor searching for genes associated with programmed cell death, or apoptosis in 1992 (12). Subsequently, Nishimura and colleagues developed the PD-1 knockout mouse model (13). These mice grow normally but develop moderate splenomegaly. Unlike the CTLA-4 knockout mouse model, the PD-1 knockout mouse survives (14). Their proliferative B cell response is augmented along with increased serum levels of certain immunoglobulins (13). PD-1 deficient mice also develop a number of autoimmune diseases, suggesting the very important role of this receptor in immunologic tolerance through negative regulation of proliferation and differentiation of B cells. Multiple subsequent studies confirmed the importance of the B7-H1/CD80 pathway in the induction and maintenance of tolerance in T cells (15).

Characteristics of PD-1 and PD-L1

The PD-1 protein is a co-inhibitor receptor of T cells with a similar structure to that of CTLA-4 but with different biologic function and specificity for ligands. PD-1 has two known ligands: PD-L1 (B7-H1 or CD274) and PD-L2 (B7-DC or CD273). There is greater affinity for PD-L1. Unlike the ligands of CTLA-4 CD80 (B7-1) and CD86 (B7-2), PD-L1 is selectively expressed and inducible in lymphoid, and non-lymphoid tissues; in different tumors (16) and in other cells of the tumor microenvironment, in response to inflammatory stimuli (17). The expression of PD-L2 is more limited (18). Latchman and colleagues described PD-L2, a second ligand for PD-1 and proved that inhibition of PD-L2, substantially inhibits T cell receptor (TCR)-mediated proliferation and cytokine production by CD4+ T cells. These researchers also demonstrated redundancy in the activity of these two receptors (19). The PD-1/PD-L1 pathway can be used by tumor cells for their own protection from immunological responses mediated by T cells (20,21). In fact, an increased regulation of PD-L1 is associated with decreased

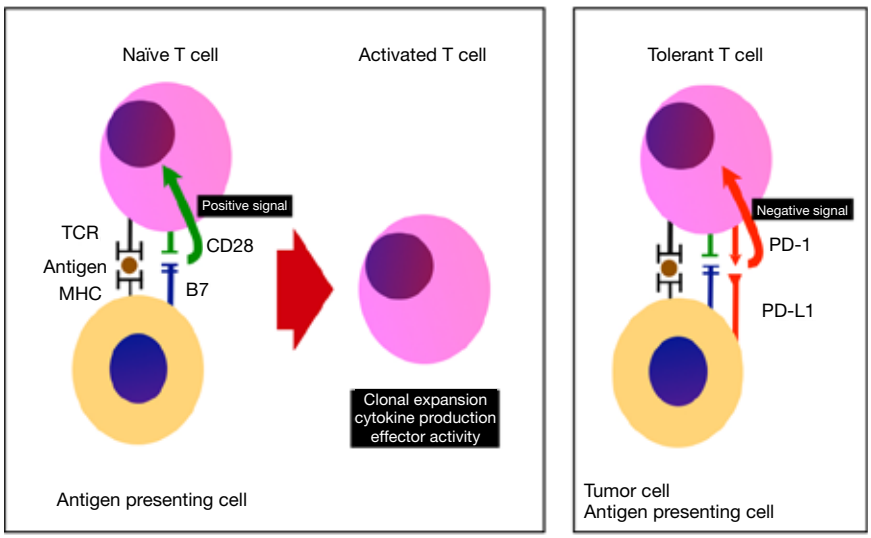

Figure 1 Stimulatory and inhibitory signals in T cell activation. Naïve T lymphocytes are introduced to an antigen by an antigen-presenting cell. This interaction occurs through the binding of the T cell receptor (lymphocyte surface) and the receptor of the major histocompatibility complex (MHC) (surface of the antigen presenting cell). The second step takes place through the binding of CD28 and its ligand B7 (CD80/86). Once this interaction occurs, the cell learns the characteristics of the antigen, secretes cytokines, clonally expands, and performs effector functions. The right panel depicts the interaction of PD-1 and PD-L1 with a resulting negative signal, and T-cell inactivation. The inhibitor antibodies of PD-1, PD-L1, or PD-L2 block the contact of these two receptors, inhibit the negative signal, and reactivate T cells.

immunological activation and adverse clinical results. The increased regulation of PD-L1 in tumor cells can inhibit the production of cytokines and cytolytic activity of PD-1(+) and tumor-infiltrating T cells with CD4(+) and CD8(+) surface expression. Hence, the inhibition of PD-1 and PD-L1 is known to enhance the immune responses *in vitro* and mediate anti-tumor activity in animals (22) and humans (*Figure 1*).

Expression of PD-L1 in human malignancies

The immunohistochemistry expression of PD-L1 by cancer cells varies substantially (23). PD-L1 is overexpressed on the surface of non-small cell lung cancer (NSCLC) cells between 21–95%; melanoma 38–100%; kidney cancer 14–44%; bladder 20–28%; head and neck 31–66%; breast 18–50%; thymic carcinoma 88-100%; multiple myeloma 93% (23). Several groups of investigators have reported worse clinical outcomes among a variety of patients with PD-L1(+) expressing malignancies (24-28). Recently, Zhang and others reported a meta-analysis confirming an adverse prognosis associated with the expression of PD-L1 on tumor cells and PD-1 on tumor-infiltrating lymphocytes (TIL) by immunohistochemistry in patients with epithelial-originating malignancies (29). The investigators found a significantly poorer survival among patients with PD-L1(+) epithelial malignancies compared with those with PD-L1(–) tumor tissues (HR 1.81; 95% CI, 1.33–2.46; P<0.001). Similarly, patients with PD-1(+) TILs had significantly shorter overall survival than the PD-1(–) group (HR 2.53; 95% CI, 1.22–5.21; P=0.012). Furthermore, all subgroups with PD-L1(+) tumors showed consistent trends toward unfavorable prognoses regardless of the assay utilized for the evaluation of PD-L1. The expression of PD-L1 has also been studied in hematological malignancies including Hodgkin's disease, non-Hodgkin's lymphomas, and multiple myeloma (30-33). In classic Hodgkin's lymphoma, alterations in chromosome 9p24.1 increase the abundance of the PD-1 ligands, PD-L1 and PDL2, and promote their induction through Janus kinase (JAK) signal transducer and activator of transcription (STAT) signaling. Early responses in patients with Hodgkin's disease led to a clinical trial for patients with relapsed or refractory disease (ClinicalTrials.gov number, NCT01592370). Twenty of 23 patients

Table 1 PD-1 pathway agents under development

Target	Agent	Structure	Manufacturer	Indication
PD-1	Nivolumab	Human IgG4 kappa	Bristol-Myers Squibb; Ono pharmaceutical, Co.	Melanoma; NSCLC; head & neck
	Pembrolizumab	Humanized IgG4	Merck & Co.	Melanoma; NSCLC
	Pidilizumab	Humanized IgG1	CureTech Ltd	DLBCL; melanoma
	AMP-514	Humanized IgG4	MedImmune	Advanced malignancies
	AUNP-12	Peptide Agonist	Aurigene Pierre Fabre	Advanced malignancies
PD-L1	BMS-936559	Human IgG4	Bristol-Myers Squibb	Advanced malignancies
	Atezolizumab	Human IgG1	Genentech & Roche	Bladder; NSCLC
	Durvalumab	Humanized IgG1	Astra Zeneca	Glioblastoma; head and neck; NSCLC; colorectal; bladder
	Avelumab	Fully Humanized IgG1	Merck KGaA EMD Serono, Pfizer	Advanced malignancies; NSCLC
PD-L2	AMP-224	PD-L2 IgG2a fusion protein	Amplimmune	Advanced malignancies

Modified and adapted with permission from Ma *et al*. (35). PD-1, programmed death-1; NSCLC, non-small cell lung cancer; DLBCL, diffuse large B cell lymphoma.

attained objective responses (4 complete responses and 16 partial responses). The rate of progression-free survival at 24 weeks was 86% (95% CI, 62% to 95%). The median survival for responders had not been reached after 40 weeks of follow-up (34).

PD-1 and PD-L1 inhibition as cancer therapies

Ten monoclonal antibodies with high affinity for PD-1 or its ligands are under development (*Table 1*). Extensive basic and clinical research has demonstrated important signals of anti-tumor activity in several tumor types. As of July 2016, this group of agents has received approval in combination with ipilimumab for the treatment of patients with BRAF V600 wild-type, unresectable or metastatic melanoma (nivolumab) or unresectable or metastatic melanoma and disease progression following ipilimumab and, if BRAF V600 mutation positive, a BRAF inhibitor (pembrolizumab) (36-41), metastatic squamous NSCLC with progression on or after platinum-based chemotherapy (nivolumab) (42,43), or metastatic NSCLC whose tumors express PD-L1 as determined by an FDA-approved test, with disease progression on or after platinum-containing chemotherapy (pembrolizumab) (44), advanced renal cell carcinoma (nivolumab), in patients who have received prior anti-angiogenic therapy (45,46), locally advanced or metastatic urothelial carcinoma (atezolizumab) who have experienced disease progression during or following platinum-containing chemotherapy or have disease progression within 12 months of neoadjuvant or adjuvant treatment with platinum-containing chemotherapy (47), and patients with Hodgkin's disease (nivolumab) that have relapsed or progressed after autologous hematopoietic stem cell transplantation (HSCT) and post-transplantation treatment with brentuximab vedotin (Adcetris) (34). Approval for recurrent head and neck carcinoma is expected before the end of 2016 and submissions for other indications are underway. While a major advance in cancer medicine, treatment with this group of agents is associated with major objective responses in a limited group of patients—similar to traditional cytotoxics; and much like chemotherapy, the combination of these agents result in greater toxicity (38). Nonetheless, in contrast with traditional cytotoxic chemotherapy, the responses induced by checkpoint inhibitors can be long lasting, and occasionally, major anti-tumor responses may follow long-term stabilization of tumor growth (48).

Biological markers

The U. S. Food and Drug Administration approved 18 new cancer agents in 2015, and most of them corresponded to targeted therapies. Predictive biomarkers for these therapies are aimed at selecting subgroups of patients with

the greatest likelihood of benefit while sparing others from unnecessary expenditures and toxicities. Hence, the development of specific biomarkers concomitantly with these agents has become more important than ever before (49). Ascertaining biological markers for immune modulating therapies are particularly challenging due to our limited knowledge of the immune system and its dynamic interactions with the microenvironment and other cellular structures. These limitations have led to a lack of uniform standardization, quantification, and interpretation of predictive biomarkers in immunology (50).

As previously discussed, while PD-L1 is widely expressed among different tumor tissues, PD-L2 is more often restricted to immune cells. Therefore, the immunohistochemical evaluation of PD-L1 expression on tumor cells has become the most accepted predictive biomarker for PD-1/PD-L1 blocking therapies. It is generally accepted that high tumor expression of PD-L1 correlates with greater response rates, duration of response, and overall survival (44). However, a considerable proportion of PD-L1(–) patients experience substantial anti-tumor responses and significant differences in survival according to PD-L1 status have not been uniformly observed across all clinical trials. Hence, a universal acceptance and application of this marker remains controversial (42,45,51). In an attempt to further evaluate the prognostic value of PD-L1 expression, Aguiar and collaborators studied the records of 1979 patients with NSCLC enrolled in 13 clinical trials using Cochrane methodology (52). The investigators found a 29% response rate among 652 PD-L1(+) patients. In contrast, a 13% response rate was found among 915 patients with PD-L1(–) tumor samples (RR 2.08; 95% CI, 1.49–2.91; P<0.01). In addition to confirming an association between overall response rate (ORR) and PD-L1 status, there was an association with the intensity of PD-L1 expression independently of the immunohistochemistry assay utilized in the study (i.e., DAKO 28-8, VENTANA SP142, DAKO 22C3). The 24-week progression free survival was also evaluated in 6 of the studies included in the analysis. Among them, the ORR was 35% for 358 PD-L1(+) patients and 26% for 409 PD-L1(–) patients. This difference was also statistically significant (RR 0.79; 95% CI, 0.71–0.89; P<0.01). Interestingly, the 1-year survival rates were not different. The survival rate for 617 PD-L1(+) positive patients was 28% versus 27% for 779 PD-L1(–) patients. The heterogeneity of the groups was substantial and the difference did not reach statistical difference (RR 0.96; 95% CI, 0.87–1.06; P=0.39).

To add further complexity to the evaluation of PD-L1 expression as a biomarker, the biologicals currently under development have adopted different methodologies and cut-off points. An example of the conflicting preliminary results in this respect, is the FDA approval of nivolumab for the treatment of patients with metastatic NSCLC regardless of their PD-L1 status whereas the approval of pembrolizumab for the same patient population was limited to those with positive PD-L1 expression on their tumor tissues based on data submitted by the sponsor of the trial demonstrating superior efficacy among patients who expressed PD-L1 >50% using a tumor proportion score (53). Some of the challenges posed by this biomarker have been described and include: technical differences challenges related to assay performance, intra-tumoral heterogeneity of biomarker expression, and dynamic changes in PD-L1 expression related to previous therapies (51,54). Several groups of investigators continue to explore other biomarkers at a cellular (CD8+ T cells) and genomic (mismatch repairs in colorectal carcinoma) to be utilized alone or in combination with PD-L1 tumor expression (55).

Safety profile

The blockade of co-stimulatory receptors/ligands involved in inhibition of T cell activation is critical to overcome immunological tolerance. Therefore, it is intuitive to find a myriad of immune related adverse events associated with the safety profile associated with these agents. Moreover, these toxicities are of greater incidence and intensity in regimens combining checkpoint inhibitors such as an anti-CTLA4 antibody (ipilimumab) with PD-1 or PD-L1 antibodies (38).

Eigentler, Hassel, *et al*. comprehensively reviewed the safety profile and current recommendations for the treatment of immune related adverse events associated with PD-1 and PD-L1 blockade (56). Their manuscript is a comprehensive description of toxicities observed during the development of PD-1 and PD-L1 inhibitors, and thoroughly visits each major group of endocrinopathies describing the time to presentation after treatment, their incidence in different clinical trials, and most importantly, the current treatment recommendations according to the type and severity of the adverse event.

For the most part, the toxicities associated with PD-1/PD-L1 inhibitors are grade 1 or 2 and easily manageable. Grade 3 or 4 toxicities are observed in approximately

1–3% of all patients. It is common practice to treat all grade 2 adverse events with corticosteroids (prednisone or methylprednisolone 1 mg/kg) until improvement to grade 1 or complete resolution. Therapy with the PD-1/PD-L1 inhibitor may continue thereafter once the steroids are tapered. Grade 3 or 4 toxicities also require systemic steroid therapy. However, on occasion, different types of adverse events may require higher doses of steroids, or the use of more potent immune suppressants. In these cases, and with the exception of hypothyroidism—in which thyroid supplementation is implemented, permanent discontinuation of the PD-1 inhibitor is recommended.

Discussion

The discovery of Immune checkpoint inhibitors constitutes the greatest historical advancement in the field of immunotherapy. Their importance is several folds. By renewing enthusiasm in the field of immunotherapy, immune checkpoint inhibitors have attracted major attention of researchers, clinicians, patients, and public in general. Such attention has resulted in large financial investments and established immuno-oncology as a solid field of cancer medicine.

Approximately ten different agents are being developed against different malignancies with promising preliminary results. The U.S. FDA has granted approval for five different tumor types including melanoma, NSCLC, renal cell carcinoma, bladder cancer, and Hodgkin's disease. Several other indications are in the process of completing their developing pathways and await or are under review by U.S. Federal authorities for approval. Current data available demonstrate improved safety and efficacy over selected traditional cytotoxics (43) and excellent efficacy in front line, or as second (39) or third line options (34) in different tumor types. Furthermore, fewer patients experience grade 3 or 4 toxicities when compared with traditional chemotherapy. In a majority of patients, toxicities are mild and manageable. Patients with moderate to severe toxicities require corticosteroids and their outcome is good for the most part (56). The development of other novel therapeutics with different mechanisms of action in immuno-oncology will rapidly demonstrate toxicity and safety signals allowing us to incorporate them to the management of patients with different types of solid and hematological malignancies. This rapid explosion of available options will soon lead to training programs in the field of immuno-oncology around the globe and accelerate future discoveries.

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Footnote

Conflicts of Interest: Dr. Camacho is a member of the bureau of speakers for Merck Inc. and has served as member in several advisory boards for Merck Inc., Bristol Myers Squibb, and Amgen. Dr. Camacho also served as principal investigator for Pembrolizumab, Avelumab, and MDX-1105.

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A new addition to the PD-1 checkpoint inhibitors for non-small cell lung cancer—the anti-PD-L1 antibody—MEDI4736

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It has been over two years since the phase I studies of the programmed death-1 (PD-1) checkpoint inhibitors—antibodies against PD-1 and its ligand PD-L1—were first presented and published internationally, demonstrating prolonged tumor regressions and improvements in survival (1,2). These results created a paradigm shift in the field of immunotherapy, in that responses were not only observed in melanoma and renal cell cancer, but also in cancers not historically thought to be immunogenic, including lung and ovarian cancer. The past few years have witnessed the rapid development of these agents into phase III registration trials. As shown in *Table 1*, over seven pharmaceutical companies are now racing to develop and obtain indication of a variety of PD-1 checkpoint inhibitors in a variety of solid tumors. Recently, FDA approval was granted to pembrolizumab (MK-3475) as the first PD-1 checkpoint inhibitor for advanced melanoma, after progression on the cytotoxic T lymphocyte antigen-4 (CTLA-4) inhibitor, ipilimumab (3). However, as the development has been so rapid, there is still little known about the differences between the anti-PD-1 and anti-PD-L1 agents developed by the various companies. Immune-related toxicity management algorithms remain in development and the use of immune response criteria is still evolving. Nevertheless, the tolerability and responses seen by these agents have been remarkable. Today, updated phase I trial results for the anti-PD-1 antibody nivolumab (4), the anti-PD-L1 antibody MPDL3280A (5), and the anti-PD-1 antibody pembrolizumab (MK3475) (6) leave little lingering doubt that immune checkpoint blockade achieves meaningful and lasting responses in cancer patients, and the list of promising PD-1/PD-L1 inhibitors as well as the list of different tumor types responding to these agents continue to grow.

A relative newcomer to the field, MEDI4736, has now joined the rapidly expanding body of PD-1 data, with the presentation of its preliminary results from a recent phase 1 study with expansion cohorts in solid tumors (7,8). MEDI4736, a fully-human anti-PD-L1 antibody engineered with a triple mutation in its Fc domain to remove antibody-dependent cell-mediated cytotoxicity, demonstrated an overall response rate across all tumor types of 11% (9/179), 22% among PD-L1+ patients (8/37), and 4% among PD-L1 negative patients (5/113). Within the lung cancer cohort, the overall response was 13%, but up to 39% in PD-L1+ patients and 5% in PD-L1 patients. Based on these outcomes in lung cancer, which aligned with the promising lung cancer data from the other PD-1/PD-L1 phase 1 studies (ORR range, 10-23%), MEDI4736 entered a multi-center, international phase III trial earlier this year for combination therapy with chemoradiation in stage III, unresectable non-small cell lung cancer (clinicaltrials.gov identifier NCT02125461) and a phase II/III trial for recurrent stage IIIB and IV squamous cell lung cancer (NCT02154490).

Preliminary results from the MEDI4736 phase 1 trial in head and neck cancer were also recently presented, showing tumor shrinkage in 7 of 29 evaluable, heavily-pretreated head and neck patients, with none of the responders experiencing relapse yet at 6-24 weeks of follow up (9). Among squamous cell carcinoma of the head and neck, the response rate was 14% (3/22), 50% of PD-L1+ patients (2/4) and 6% (1/16) of PD-L1 negative patients. Responses were seen in other tumor types, but the data is not mature. The disease control rate (RECIST response + stable disease ≥12 weeks) for all tumor types was 31%, 54% (20/37) for PD-L1+ patients, and 21% (24/113) for PD-L1 negative patients, which supports the argument that our traditional measure of response rate does not capture the full efficacy of the checkpoint inhibitors.

MEDI4736 shares many of the hallmarks of its PD-1

Table 1 Seven companies developing PD-1 checkpoint inhibitors

Drug	Company	Isotype	Clinical development
Anti-PD-1 antibodies			
Nivolumab	Bristol-Myers Squibb	IgG4	Phase 3
MK-3475	Merck & Co	IgG4 (humanized)	Phase 3
Pidilizumab	CureTech	IgG1 (humanized)	Phase 2
AMP-224	AstraZeneca/Medimmune	PD-1/B7 Fc fusion protein	Phase I
AMP-514	AstraZeneca/Medimmune	IgG	Phase I
	Novartis (CoStim)	IgG	Phase I
Anti-PD-L1 antibodies			
MPDL3280A	Genentech/Roche	IgG1	Phase 3
MEDI-4736	AstraZenaca/Medimmune	IgG1	Phase 2
MSB0010718C	EMD Serono (Merck KGa)	IgG	Phase 1

competitors: activity across multiple tumor types, rapidity of response with many tumor regressions observed at the first 6-week restaging study, and—most importantly—durability of response, lasting as long as 67 weeks without ongoing treatment. While the PD-L1 status correlated with higher response rates in a variety of tumor types, there were also meaningful responses observed in PD-L1 negative patients, as has been seen in other trials with different PD-1 inhibitors, limiting the reliability of PD-L1 status to determine who should be treated with these agents. It is not yet known if this is due primarily to heterogeneity and nonstandardized methods of immunohistochemical and molecular methods for the current testing for PD-L1 (varying cut-off criteria for positivity, PD-L1 status of infiltrating tumor lymphocytes, stromal or tumor cells and specific antibodies used), or if this is due to the unreliability of PD-L1 status itself, which may change *in vivo* in response to different environmental contexts as well as vary across different tumor sites in the same patient (10).

MEDI4736 may distinguish itself slightly from the other anti-PD-1 agents by its tolerability. Although it is difficult to draw meaningful conclusions from differences across separate trials of the various PD-1 drugs, the toxicities of MEDI4736 were minimally lower than what has been reported with other agents, with only 6% grade 3/4 adverse events in the expansion cohorts, notably no colitis, and only one event of grade 2 pneumonitis, which was reversible (7). MEDI4736 also demonstrated low immunogenicity, with only a 3% incidence (1 in 32 patients) of positive anti-drug antibodies that impacted pharmacokinetic and pharmacodynamics levels (11). This may be a result of MEDI4736 being a fully human antibody, compared to other PD-1 antibodies that contain humanized murine regions which can contribute to variations in affinity and immunogenicity among the PD-1 antibodies. Moreover, it is not known whether the use of an IgG1 or IgG4 backbone elicits any differences in efficacy or tolerability.

The differences between anti-PD-1 antibodies versus those of the anti-PD-L1 antibodies are not well elucidated either. As antibodies specifically targeting PD-L1 such as MPDL3280A and MEDI4736 only block the PD-1: PD-L1 interaction, these agents could theoretically lead to less toxicity than the anti-PD-1 drugs which block both PD-1: PD-L1 and PD-1: PD-L2 binding (12,13). Seemingly minor differences in toxicity patterns become very relevant when considering the distinct tumor types and patient populations that are being targeted for PD-1 development. The toxicity of pneumonitis, for example, can be particularly life threatening in lung cancer where patients often already have compromised lung function from smoking, COPD, radiation history, as well as the cancer itself. However, there is also evidence that some tumors, such as esophageal, hepatocellular and ovarian, express PD-L2, in which case an anti-PD-1 antibody that binds to both PD-L1 and PD-L2 may prove to be more toxic but also more effective (14). It appears some of these antibodies may demonstrate greater efficacy in specific tumor types as opposed to others. For example, MPDL3280A has distinguished itself by its tolerability and efficacy in NSCLC and bladder cancer. It is currently in phase II and III registration trials in lung cancer (NCT02031458, NCT02008227) and in phase II trials in bladder cancer (NCT02108652).

With the presentation of data from each new, successive PD-1 agent, the question remains as to whether the latest PD-1 inhibitor will distinguish itself from its predecessors, and whether one particular agent will prove to be the "winner" in efficacy and side effect profile. MEDI4736 has established itself as a clear contender in the growing family of well-tolerated and promising PD-1 inhibitors, and has entered accelerated development into phase III trials for lung cancer. While there hasn't been a head-to-head comparison, its preliminary response rates appear at least comparable to the responses observed with other PD-1 inhibitors. It remains to be seen whether the results of these different PD-1 drugs—while promising—reflect a redundancy in anti-tumor activity or whether they will offer a unique therapeutic profile. As we develop a better understanding of the impact of the varying structural elements of these antibodies, and the interactions between PD-1 and PD-L1/L2 on different tumor types, we may find there is not a single winner, but rather that different PD-1 checkpoint inhibitors work better in certain tumor types and for certain patients. As the mechanisms of resistance are investigated, a role may also emerge for the use of these agents in combination with each other and other compounds. For now, the race continues.

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PD-L1 expression in lung cancer

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It is with great interest that we read the recent manuscript written by Dr. Yu and colleagues in the *Journal of Thoracic Oncology* (1). In this manuscript Yu *et al.* describes different aspects of PD-L1 expression both at the protein and mRNA level in two cohorts of small cell lung cancer (SCLC) patients (1). These data are especially timely given the recent publication of Checkmate 032, a phase I/II trial of nivolumab and nivolumab plus ipilimumab in patients with recurrent small-cell lung cancer (2). Their data provides further clarification regarding PD-L1 pattern in SCLC, a cancer that has not been extensively treated with immunotherapy agents, but for which there is significant biologic rationale given the high mutation burden (3).

The impetus for PD-L1 staining is driven by recent non-small cell lung cancer (NSCLC) trials that demonstrated associated with endpoints including objective response, progression free survival, and overall survival (4-7). Partially as a result, percent tumor PD-L1 expression level has arisen as an inclusion criteria for the most recent randomized studies comparing PD-1 inhibitors to standard of care chemotherapy as first or second line therapy of metastatic NSCLC (8,9). Further corroborating these results are recent data from KEYNOTE-141 where PD-L1 expression was associated with an overall survival benefit for nivolumab treatment (10). Despite these associations in NSCLC and head and neck cancer, data in other histology have yielded mixed results. Trials in melanoma and renal cell carcinoma have not demonstrated significant associations between patient outcomes and PD-L1 expression (11-13). To explain the heterogeneity of the observed association between PD-L1 expression and clinical response, we feel an often overlooked aspect is technical details of the tumor biopsy and PD-L1 staining. In our experience there are significant differences in PD-L1 staining when scoring different size biopsy samples (unpublished data). We attribute this to the variable spatial expression patterns of surface PD-L1 expression on tumor cells. We have observed that PD-L1 expression occurs in isolated clusters or band-like "expression fronts". When such regions of high PD-L1 density are by chance sampled, higher PD-L1 expression is recorded than if a larger biopsy (or whole tumor sections) were to sample areas of both high and low PD-L1 expression. Furthermore, variations also exist in staining technique, biopsy timing and technique, organ site biopsied, and varying anti-PD-L1 antibodies. It is therefore of particular relevance that Yu *et al.* investigates the variability in PD-L1 staining with different antibody clones and PD-L1 expression at the mRNA level (1).

Current interest in treating SCLC with immunotherapy agents stems from the high number of somatic mutations that characterize this cancer (3). The association of somatic mutation with disease response to pembrolizumab was demonstrated in a phase 2 study of patients treated for mismatch repair deficient cancers (14). This study found a high objective response rate of 53%. Of note, both this trial (14) and others (15,16) observed a significant association between a high somatic mutation load and response. Despite this, the objective response rate of SCLC patients in Checkmate 032 with nivolumab alone was modest (10%), with tumor responses occurring irrespective of PD-L1 status (2). More

encouraging were the objective response rates of patients treated with combination ipilimumab and nivolumab (22%). It is worth noting that the rate of grade 3–4 toxicity associated with this combination therapy were not as high as those observed in earlier trials treating melanoma with combination immunotherapy (17). To further explore the role of immunotherapy for SCLC, our department is conducting an investigator-initiated phase I study to assess the effects of pembrolizumab and radiation in extensive and limited stage disease (NCT02402920). The rationale behind such a treatment paradigm is that radiation releases antigens, providing greater immune system access to the array of somatic mutations inherent in this disease (18,19).

In conclusion, although PD-L1 testing is fast emerging as standard test in to select immunotherapy treatment for NSCLC, whether such a test exhibits utility in SCLC remains to be determined. The analysis conducted by Yu *et al.* provides further insight into PD-L1 testing and expression levels for SCLC (1), a valuable addition to the literature especially as data on immunotherapy treatment for SCLC emerges. Finally, we stress that although strong biologic rationale exists for immunotherapy selection based on PD-L1 staining, variability in staining and biopsy samples may produce a level of inter-sample variability that makes these associations difficult to identify.

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Footnote

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Reflections on immune checkpoint inhibition in non-small cell lung cancer

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Introduction

Despite the expanding armamentarium of treatment modalities against metastatic non-small cell lung cancer (NSCLC) this disease remains incurable. The introduction of targeted therapies provides transient control of disease for some molecular subtypes but virtually all patients' progress. Recently immunotherapies have been approved for the treatment of metastatic melanoma and prostate cancer, and have shown promise for the treatment of NSCLC. More specifically, the blockade of immune checkpoints may improve outcomes in the treatment of NSCLC. Immune checkpoints modulate immune responses to effectively balance self-tolerance and tissue destruction. Many tumors express immune checkpoints or their ligands to inhibit anti-tumor immune responses. One of the most important immune checkpoints is programmed cell death ligand 1 (PD-L1), which was discovered at Mayo Clinic (1). PD-L1 was first named B7-H1 due to its homology with the B7 family of co-stimulatory molecules. When PD-L1 was initially discovered in peripheral blood monocytes, it was shown to negatively regulate T cells through IL-10 production after ligation. It was later shown that PD-L1 negatively regulated T cell proliferation through engagement of programmed cell death protein 1 (PD-1), and it has since been called PD-L1 by many groups. Additional work demonstrated that PD-L1 induces apoptosis of tumor specific T-cells (2). The expression of PD-L1 in at least a quarter of patients with NSCLC suggests that blockade of the PD-1/PD-L1 axis may be effective therapy for NSCLC (3).

Pharmaceutical companies have developed antibodies that block PD-1 or its ligand PD-L1. There was noteworthy clinical activity in one of the first phase I dose-escalation clinical trials with a fully humanized IgG4 anti-PD-1 antibody. This trial included patients with metastatic colorectal cancer, renal cell carcinoma, melanoma, castrate-resistant prostate cancer and NSCLC. Out of 39 patients, one complete response and two partial responses were observed in addition to some mixed responses. Successful treatment was associated with tumor cell surface expression of PD-L1 and significant increases in lymphocyte infiltration into metastatic tumors (4).

PD-1 inhibition with MK-3475 in NSCLC

There has since been an increase in clinical trials targeting the PD-1/PD-L1 axis, especially in NSCLC. At the Annual Meeting of the American Society of Clinical Oncology (ASCO) 2014, an update of one such trial was presented: "safety and clinical activity of MK-3475 in previously treated patients with NSCLC" (5). MK-3475 (also known as pembrolizumab) is a humanized monoclonal IgG4 antibody against PD-1 and was recently approved by FDA for the treatment of patients with unresectable or metastatic melanoma and disease progression following ipilimumab and a BRAF inhibitor if a BRAF V600 mutation is present. In this clinical trial, previously-treated patients with NSCLC whose tumors expressed any detectable PD-L1 by an immunohistochemical assay were randomized to receive MK-3475 at 10 mg/kg every 2 weeks or every 3 weeks. Some patients who had received 2 or more prior lines of therapy and whose tumors did not express PD-L1 were also treated with MK-3475 at 10 mg/kg every 2 weeks. Responses were determined by investigators using

immune-related response criteria (irRC) in addition to an independent central review using Response Evaluation Criteria in Solid Tumors 1.1. Out of 450 patients who provided tissue, 305 patients' tumors expressed PD-L1 with the antibody that was utilized for immunohistochemistry (68%). Of the 221 patients who were treated with MK-3475, 6% of patients experienced grade 3-4 adverse events, and 48% of patients experienced grade 1-2 adverse events. The most common events were fatigue and decreased appetite, although a few developed pneumonitis. The preliminary confirmed overall response rate was 15% (16% for tumors that expressed PD-L1 and 10% for patients whose tumors did not express PD-L1). The confirmed response rate was 19% for patients treated at 10 mg/kg every 2 weeks, and 15% for patients treated at 10 mg/kg every 3 weeks. Many of the patients were still on treatment at the time of the presentation.

These are very promising results in this developing era of immunomodulating agents for the treatment of NSCLC. First of all, MK-3475 seems relatively well tolerated. The most concerning events were the cases of pneumonitis seen in just over 1% of patients. Thus, MK-3475 may pose a risk of pneumonitis similar to that seen with erlotinib. The 15% response rate in a heavily pre-treated patient population is encouraging for a novel therapy and holds promise for use in a subsequent line of therapy strategy. The ongoing clinical trial comparing MK-3475 to docetaxel after prior treatment with a platinum doublet will be critical to defining the role of PD-1 blockade in second line therapy for patients with NSCLC whose tumors express PD-L1 (NCT01905657).

Detection of PD-L1 expression

There are a number of issues that will hopefully be clarified in the clinical trials moving forward. The presented data suggest that there is a relationship between PD-L1 expression on tumor cell surfaces and objective responses; however 10% of patients whose tumors did not express PD-L1 responded to MK-3475. These responses could possibly be related to tumor heterogeneity, meaning the biopsied tumor did not express PD-L1 but non-sampled areas of tumor possibly did. Alternatives to small biopsies may need to be considered for determination of PD-L1 status. In this regard, some groups are investigating circulating markers of PD-L1 expression by tumors, such as our efforts to determine if downstream signaling of CD8+/PD-1+ T cells predict engagement with PD-L1 (6). Additionally, the 68% of patients with PD-L1 expression in this clinical trial is higher than that of other reports (4). Questions have been raised about the validity of the currently used immunohistochemical assays. Indeed, evaluation of PD-L1 expression in NSCLC samples using multiple validated antibodies that target different PD-L1 domains produced discordant patterns of expression (7). These findings could possibly be due to different antibody affinities, cross reactivity, or variable expression of distinct target epitopes. Another study compared PD-L1 expression by immunohistochemistry to in situ hybridization (ISH) in squamous cell carcinoma of the lung, and found a higher percentage of PD-L1 expression in tumors by ISH (8). Tumor heterogeneity in NSCLC (9) may confound the importance of biomarkers, and obfuscate markers of treatment selection. A better understanding of how to select patients for immune checkpoint inhibition is needed.

Dynamics of PD-L1 expression

The expression of PD-L1 under normal circumstances is dynamic and influenced by cytokines such as interferon-γ. Recent studies suggest that PD-L1 expression by tumor cells can be influenced by cytotoxic agents and targeted therapies (10). Thus, the timing of the biopsy to determine PD-L1 expression may be critical to patient selection. Additionally, as we learn how treatments modulate PD-L1 expression we may begin to test for optimal sequences or combinations of immune checkpoint inhibitors and other approved or experimental therapies. For example, another anti-PD-1 antibody is being studied as front line therapy for patients with metastatic NSCLC whose tumors express PD-L1 (NCT02041533), and a separate trial is combining anti-PD-1 therapy with an immune checkpoint inhibitor against LAG-3 (NCT01968109).

Response evaluation with immunotherapeutics

The use of standard response criteria can be challenging with immunotherapies. Some immune checkpoint inhibitors promote infiltration of tumors with lymphocytes which is associated with a delayed response, but radiographically may present as progressive disease. It is difficult for clinicians to distinguish tumor progression from immune infiltration. Although this issue seems to affect a minority of patients, clinicians and patients would likely prefer to continue therapy resulting in immune infiltration and a likely subsequent response. Some novel imaging technologies,

such as technetium-linked IL-2 single-photon emission computed tomography are being explored in the setting of patients with metastatic melanoma receiving a different immune checkpoint inhibitor against CTLA-4, ipilimumab (NCT01789827). Detection of IL-2 receptor in this setting may help distinguish immune infiltration from tumor progression. Accordingly, this strategy may complement standard imaging modalities in the setting of progression on immune checkpoint inhibitors, providing a rationale to continue with immunotherapy when IL-2 is detected at tumor sites.

Conclusions

Immune checkpoint blockade is a very promising development for the treatment of metastatic NSCLC. We still have much to learn about PD-L1 expression, the detection of PD-L1, the selection of patients for anti-PD-1/PD-L1 therapies, the optimal combination or sequences of therapies, and the use of novel imaging modalities for the appropriate definition of responses.

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Footnote

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PD-1 targeted Immunotherapy as first-line therapy for advanced non-small-cell lung cancer patients

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The better understanding of interactions between tumor and immune system (e.g., tumor-associated upregulation of PD-L1 to induce checkpoint for cytotoxic lymphocytes; *Figure 1A*) gave rise to the development of immune modulating therapies. To overcome T-cell tolerance and to boost cellular immune response, the application of monoclonal antibodies targeting CTLA-4 (4) or the PD-1/PD-L1 axis enlarged our therapeutic options for advanced cancer patients. With regard to this type of immunotherapy, non-small cell lung cancer (NSCLC) proved to be a suitable entity. The approval of immune checkpoint inhibitors [i.e., Nivolumab (5,6), Pembrolizumab (7-9) and Atezolizumab (10)] enriched our therapeutic armamentarium for advanced NSCLC patients (11).

Basically, there are two molecular pathways with impact on tumor cell survival. On the one hand, concomitant engagement of both the T-cell receptor (TCR) and the PD-1 receptor regulate T cellular differentiation (*Figure 1B*) (3). On the other hand the binding of PD-1 to PD-L1 impairs i.a. *Fas*-mediated apoptotic mechanisms (*Figure 1C*) (2). However, PD-L1 activity is not stable. Besides tumor heterogeneity it depends on other factors such as EGFR-mutational status (12), JAK2 gene amplification (13) or following tyrosine kinase inhibitor (TKI) therapies (14).

At present, there are two PD-1 inhibiting antibodies approved for NSCLC therapy: Nivolumab and Pembrolizumab. The design of the underlying studies pivotal for approval differs in terms of methodology. Whereas Nivolumab was tested as a 2nd line treatment in squamous (5) and non-squamous (6) NSCLC patients (against prior standard of care: docetaxel chemotherapy), the KEYNOTE-001 trial focused on the response rates of Pembrolizumab in correlation with PD-L1 expression in NSCLC tumor tissues (7). Similar to the pivotal studies for Nivolumab, Pembrolizumab was compared to docetaxel chemotherapy as second line treatment in a phase II/III-study (8). Only patients with a PD-L1 expression level of at least 50% in the investigated tumor cells were included in this study (8).

Against the background of the reported favorable therapeutic results (8), Reck *et al.* investigated Pembrolizumab as 1st line treatment for untreated advanced NSCLC with PD-L1 expression on at least 50% of tumor cells. With focus on progression-free survival (as primary end point), overall survival, response rate, and safety (as secondary end points), Pembrolizumab (tested in n=154 patients with a PD-L1 expression) was compared to standard chemotherapy (n=151 patients) (9).

In the comparative analysis, Pembrolizumab was associated with increased progression free survival (PFS) [hazard ratio (HR) for disease progression or death =0.50; P<0.001], overall survival (OS) (HR for death =0.6; P=0.005) and improved response rates (44.8% *vs.* 27.8%). Moreover, less adverse events were observed for the Pembrolizumab treatment. With regard to the immune-mediated specific side effects, hypothyroidism (9.1%), hyperthyroidism

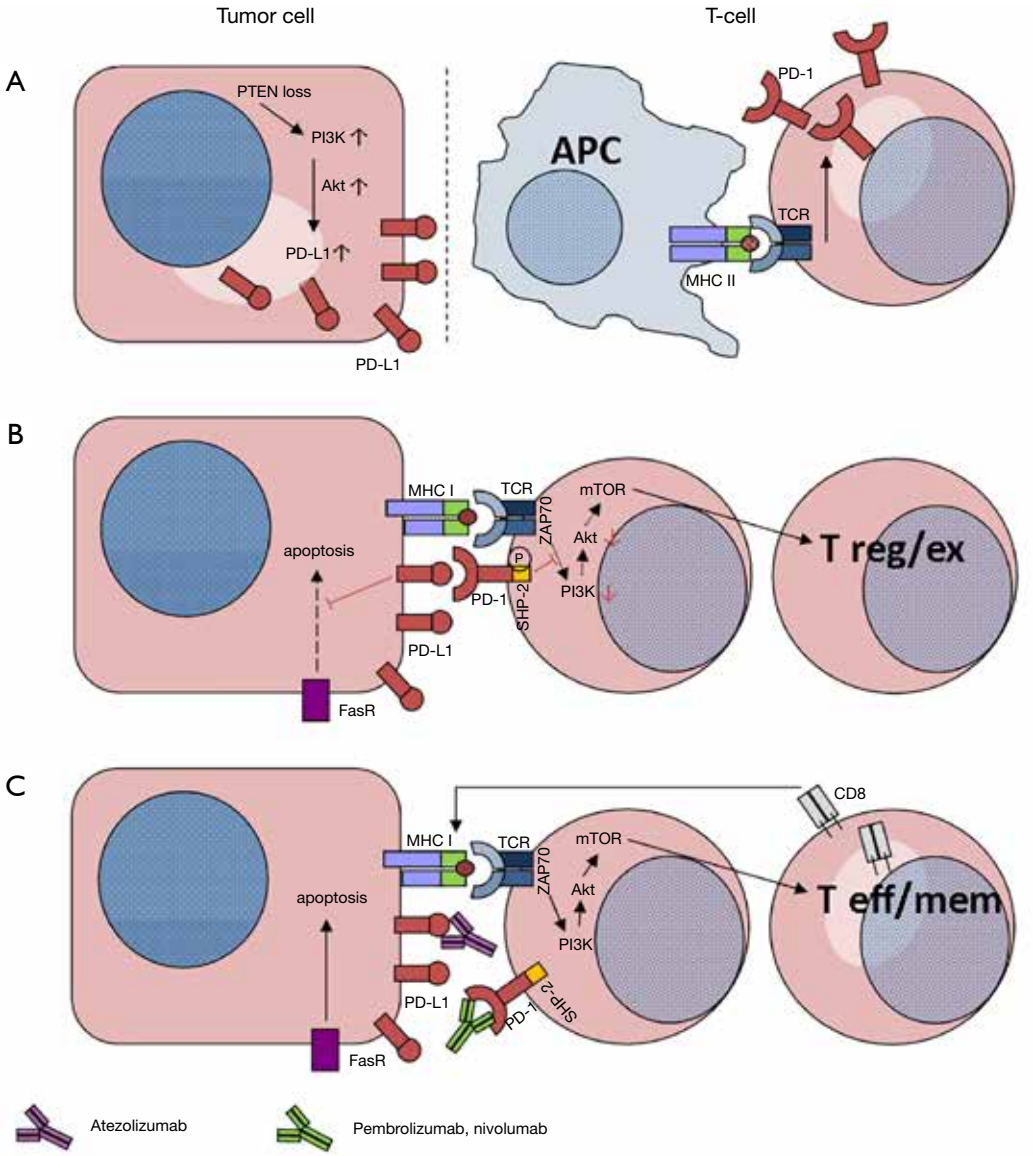

Figure 1 PD-1:PD-L1 mechanisms between T-cells and tumor cells. (A) Loss of phosphatase and tensin homolog (PTEN) in tumor cells induces the Phosphoinositol-3-kinase (PI3K)—Akt pathway with consecutive overexpression of PD-L1 (1); (B) once PD-L1 binds to PD-1, the resulting inhibition influences tumor surveillance such as Fas-mediated apoptosis (2). By co-activation of PD-1 concomitant to TCR in T-cells, the phosphorylation of the SHP-2 domain results in a down-regulation of the PI3K-Akt pathway. This step alters the mTOR complex, which regulates T cellular differentiation (3); (C) upon immune checkpoint inhibition, either with PD-L1-mAb (Atezolizumab) or PD-1-mAbs (Pembrolizumab and Nivolumab), T-cell differentiation is switched to CD8+ T effector (T *eff*) or T memory (T *mem*) cells, inducing apoptosis by completing the major histocompatibility complex (MHC) of the tumor cells (3).

(7.8%) and pneumonitis (5.8%) were observed in the Pembrolizumab cohort. Other side effects, e.g., nausea, vomiting, fatigue, and constipation were seen more often following platinum based doublet chemotherapies (9).

Due to the confirmed favorable benefit-to-risk profile of PD-1 inhibition, this therapeutic option now becomes relevance for 1st line NSCLC treatment. With regard to future therapeutic implications, the combined application of immunotherapy and chemotherapy have already been investigated in two phase I/II-trials (15,16). Whether there

are beneficial effects for both neoadjuvant and adjuvant immune checkpoint inhibition, requires further investigation.

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Footnote

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Results of clinical trials with anti-programmed death 1/ programmed death ligand 1 inhibitors in lung cancer

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Abstract: One of the main hallmarks of cancer is the capability of evading immune destruction. In order to drive tumor progression, malignant cells are able to promote immunosuppressive mechanisms avoiding recognition and elimination. Increasing knowledge of the mechanisms of immune tolerance has led to the identification of several membrane receptors strongly implicated in this cancer feature: the immune checkpoints. Among them, programmed death 1 (PD-1) receptors and their ligands have been identified as potential targets for a new anti-cancer therapeutic approach: the use of immune-modulatory monoclonal antibodies designed to interrupt the immune escape activated by the interaction of PD-1 receptors and their ligands. Five of these antibodies are now in their late stages of clinical development and this review will summarize their up-to-date efficacy and toxicity data.

Keywords: Lung cancer; nivolumab; pembrolizumab; programmed death 1 (PD-1); programmed death ligand 1 (PD-L1)

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Introduction

Immune destruction escape is one of the main features that allow cancer cells to survive, proliferate and eventually kill our patients. In order to drive tumor progression, malignant cells acquire the ability to promote immunosuppressive mechanisms to their own benefit, avoiding recognition and elimination by the host immune system. Most solid tumors are able to establish potent immunosuppressive networks that operate locally (within the tumor mass) and systemically (in the circulation and bone marrow). The immune checkpoints are different families of membrane receptors and their ligands that are strongly implicated in this cancer feature. Among them, programmed death 1 (PD-1) receptors and their ligands have been identified as potential targets for a new anti-cancer therapeutic approach.

PD-1 are immunosuppressive receptors expressed on the surface of activated T lymphocytes or natural killer cells or their ligands that are meant to control the physiological extinction of immune responses and the maintenance of peripheral tolerance. Interaction of PD-1 with its ligands, programmed death ligands 1 and 2 (PD-L1 and PD-L2), dampens T-cell receptor signaling, leading to downregulation of T-cell activation, proliferation, and T-cell-mediated antitumor immune response (1-3). The PD-1 pathway represents one of the immune checkpoints used by tumors to suppress antitumor immunity (4). Moreover, potential tumor-reactive lymphocytes are often kept in check by PD-1 transduced signals, reflecting the ability of many cancers to express increased levels of their ligands.

Immunomodulatory monoclonal antibodies inhibit safeguard systems that are harnessed by cancer cells to establish immunological tolerance (5,6). These molecules represent a promising means to induce robust and durable

responses when employed as single agents (7,8) but also hold promise to significantly boost the efficacy of several anticancer chemo-, radio- or other targeted treatments.

The safety and efficacy of immunomodulatory monoclonal antibodies have been assessed in numerous cohorts of patients with non-small cell lung cancer (NSCLC) or other pulmonary neoplasms, as well as in virtually every other major tumor type. This review summarizes up-to-date efficacy and toxicity data of the main five PD-1/PD-L1 antibodies that are in their late stages of clinical development nowadays.

Efficacy of agents targeting PD-1

At this moment there are two main different compounds targeting PD-1: nivolumab (Opdivo) and pembrolizumab (Keytruda) are anti-PD-1 monoclonal antibodies and have recently received approval from the Food and Drug Administration (FDA) in second line treatment for NSLC patients.

Nivolumab is a genetically engineered, fully human immunoglobulin G4 monoclonal antibody specific for the human PD-1 receptor. Nivolumab binds PD-1 with high affinity on activated immune cells, preventing its interaction with PD-L1 and PD-L2 ligands, therefore reducing the inhibitory signals and augmenting the host antitumor response.

First six NSCLC patients to be treated with nivolumab were enrolled in a phase I trial that was conducted in the USA from October 2006 through June 2009 (9). This phase I study sought to determine the safety and tolerability of anti-PD-1 blockade in patients with treatment-refractory solid tumors and to preliminarily assess antitumor activity, pharmacodynamics, and immunologic correlates. The trial included a total of 39 patients with different cancer types including melanoma and renal cell carcinoma among others. Patients received a single intravenous infusion of nivolumab in dose-escalating six-patient cohorts at 0.3, 1, 3, or 10 mg/kg, followed by a 15-patient expansion cohort at 10 mg/kg. Patients with evidence of clinical benefit at 3 months were eligible for repeated therapy. Six patients in total were included in the subset of NSCLC patients and from those, one patient had a significant lesional regression not reaching partial response criteria as it was defined per protocol with the dose of 1 mg/kg.

These results encouraged further development in NSCLC so a second phase I trial with specific tumor-type pre-planned expansion cohorts was conducted between November 2008 and January 2012 (10). The objective of the trial was to evaluate the safety, anti-tumor activity, and pharmacokinetics of nivolumab. One hundred and twenty-nine patients with heavily pre-treated advanced NSCLC were enrolled to receive 1.0, 3.0 or 10.0 mg per kilogram of body weight every 2 weeks. Response was assessed after each 8-week treatment cycle. Patients received up to 12 cycles until disease progression or a complete response occurred.

In this trial objective response rate (ORR) to nivolumab monotherapy was 17% (22/129 patients), lasting for a median of 17.0 months across all doses. Eleven responses (50%) were documented at the first 8-week tumor assessment in contrast with the extended idea that immune therapies are slower to show objective lesional reductions than cytotoxic treatments. However, a subset of patients achieved delayed responses and the mechanism of this is yet to be better understood.

No differences in ORR were found regarding histological subtype, 17% and 18% in squamous *vs.* non-squamous. An additional exploratory analysis of response by smoking exposure in 80 evaluable patients found ORR was higher in patients with a smoking history of more than 5 pack-years (30%; n=66) than in those with a history of 5 pack-years or less (no responses; n=14).

Median progression-free survival (PFS) across doses was only 2.3 months but interestingly PFS rates at 6 months, 1 year, and 2 years of 33%, 22%, and 9%, respectively. In fact, median PFS of the 22 responders was 20.6 months, an unprecedented long interval for heavily pre-treated NSCLC patients. Specially intriguing is the fact that among 18 responders who discontinued nivolumab therapy for reasons other than disease progression, 50% (nine) had responses for more than 9 months after the end of therapy.

Median overall survival (OS) was 9.9 months for all 129 patients with NSCLC but in 37 patients receiving nivolumab 3 mg/kg, the dose currently being used for phase III trials, median OS was 14.9 months. Again, there were no differences in median OS and survival rates in patients with squamous and non-squamous histology.

At that point nivolumab had not only showed a good safety profile but also an impressive potential to change lung cancer natural history prolonging significantly the PFS and OS of a subset of patients. These results were also observed in melanoma and renal cell carcinoma patients so an ambitious development program named CheckMate was started. CheckMate program includes several trials meant to evaluate nivolumab treatment in different tumors, settings and combinations.

CheckMate 017 was conducted from October 2012 through December 2013 and randomly assigned 272 squamous cell lung carcinoma patients to receive nivolumab, at a dose of 3 mg/kg every 2 weeks, *vs.* docetaxel, at a dose of 75 mg/m^2 every 3 weeks (11). PD-L1 protein expression was evaluated retrospectively in pre-treatment (archival or recent) tumor-biopsy specimens.

The rate of confirmed objective response was significantly higher with nivolumab than with docetaxel (20% *vs.* 9%; P=0.008). The median PFS was 3.5 months in the nivolumab group and 2.8 months in the docetaxel group, slightly disappointing, but again, those patients that achieved responses obtained long-term PFS and OS benefits. The rate of PFS at 1 year was 21% in the nivolumab group and only 6% in the docetaxel group. The median OS was 9.2 months in the nivolumab group as compared with 6.0 months in the docetaxel group with the risk of death 41% lower with nivolumab (hazard ratio, 0.59). The OS rate at 1 year was 42% in the nivolumab group *vs.* 24% in the docetaxel group. The hazard ratios for death in the analysis of OS were favorable to nivolumab in almost all subgroups but not in those patients who were 75 years of age or older.

CheckMate 063 was conducted between November 2012 and July 2013, designed as a phase II open label, multinational and multicenter single arm trial in 117 patients (12). In this trial nivolumab was given to squamous cell lung cancer patients who had progressed at least to two lines of chemotherapy including a platinum containing doublet. Again, patients were included regardless of PD-L1 status. ORR assessed by an independent radiology review committee was 14.5% (17 patients) and median duration of response was not reached (95% CI, 8.31–not applicable); as much as 13 (76%) of 17 of responses were ongoing more than 6 months. Twenty-six percent of patients had stable disease with a median duration of 6 months. Median PFS was 1.9 months, with PFS of 20.0% at 1 year. Median OS was 8.2 months and OS at 1 year was 40.8%.

Nivolumab was FDA approved on March 2015 to treat metastatic squamous NSCLC with progression on or after treatment with platinum-based chemotherapy based on combined data from CheckMate-017 and -063.

The most recently published trial has been CheckMate 057 that was conducted from November 2012 through December 2013 to confirm if the results observed in squamous-cell lung cancer were also reproducible in the non-squamous histology subset (13). It was a phase III trial that randomized 582 patients with advanced non-squamous NSCLC after failing platinum doublet chemotherapy to nivolumab at 3 mg/kg intravenously every 2 weeks (n=292) or docetaxel (n=290). The response rate was 19% with nivolumab *vs.* 12% with docetaxel (P=0.02). Although PFS did not favor nivolumab over docetaxel (median, 2.3 months and 4.2 months, respectively), the rate of PFS at 1 year was higher with nivolumab than with docetaxel (19% and 8%, respectively) consistently with the long lasting responses that have been previously observed. In this trial conversely of what was observed in the squamous-cell lung cancer population, nivolumab was associated with even greater efficacy than docetaxel across all end points in subgroups defined according to pre-specified levels of tumor-membrane expression (≥1%, ≥5%, and ≥10%) of the PD-1 ligand.

The median OS was 12.2 months in the nivolumab group and 9.4 months in the docetaxel group (hazard ratio for death, 0.73). The OS rate at 18 months was 39% with nivolumab *vs.* 23% with docetaxel.

On October 2015 the FDA expanded the approval of nivolumab to include patients with non-squamous NSCLC on or after progression with platinum-based chemotherapy with the data from CheckMate-057.

Currently there are several key nivolumab trials ongoing to address important questions as the activity of nivolumab on different clinical settings and in combinations. Results from a phase III trial, CheckMate 026, comparing nivolumab *vs.* chemotherapy in the first line setting for PD-L1 positive NSCLC patients are pending (NCT02041533).

Preliminary results of a phase I trial of nivolumab in combination with platinum-based doublet chemotherapy have been reported (14). In this trial 56 chemotherapy-naïve patients with advanced NSCLC were assigned into four different cohorts according to histology to receive different chemotherapy regimens with nivolumab (at 5 mg/kg or 10 mg/kg doses) every 3 weeks. Treatment was given for four cycles, with continued nivolumab alone until progression or unacceptable toxicity. ORR was similar in the four cohorts and ranged from 33% to 47%. An astounding OS rate of 86% at 18 months was reported in the nivolumab combined with carboplatin and paclitaxel arm for patients of either histology (squamous and non-squamous).

Other CheckMate trials ongoing are: CheckMate 012 (NCT01454102), a phase I trial with multiple arms using combinations of nivolumab with chemotherapy, bevacizumab, Ipilimumab or erlotinib; CheckMate 227 (NCT02477826), a phase III trial to test the combination

of nivolumab plus Ipilimumab *vs.* chemotherapy in the first line setting and CheckMate 331(NCT02481830), a phase III trial for small cell lung cancer (SCLC) patients that will test nivolumab *vs.* topotecan in the second line setting.

Pembrolizumab (Keytruda) is a highly selective IgG4-κ humanized monoclonal antibody directed against human cell surface receptor PD-1 (15). Its mechanism of action is similar to nivolumab, binding PD-1 with high affinity on activated immune cells to prevent its interaction with PD-L1 and PD-L2 ligands.

The first-in-human phase 1 trial to evaluate pembrolizumab in the clinical setting was called KEYNOTE-001, and it was a large, international, multicohort study for the treatment of patients with advanced solid tumors (15,16).

Between April 27, 2011, and August 1, 2012, 32 patients were enrolled in the dose escalation, part A of the trial, in which seven NSCLC patients were included (15). Two of them showed tumor shrinkage that did not meet RECIST v1.1 objective response criteria: one patient treated with 10 mg/kg every 2 weeks (initial tumor reduction of 9.7%, followed by progression in subsequent imaging performed 8 weeks later) and one patient treated with 1 mg/kg every 2 weeks (initial 25% decrease in target lesion, followed by progression in a subsequent imaging performed 8 weeks later). Two more NSCLC patients achieved disease stabilization. Data from this part of the trial provided the basis for enrolling patients in multiple NSCLC expansion cohorts of KEYNOTE-001 part B.

From May 2012 through February 2014, a total of 495 patients received at least one dose of pembrolizumab in several cohorts in which two doses were tested: intravenous pembrolizumab at a dose of 2 mg or 10 mg per kilogram every 2 weeks over a 30-min period.

One of the main differences with the CheckMate program used to develop nivolumab was that in KEYNOTE-001 a contemporaneous biopsy sample was required to determine the PD-L1 status for eligibility.

The overall response rate was 19.4% which included a response rate of 18.0% in the 394 previously treated patients and 24.8% in the 101 previously untreated patients. The response rate was similar regardless of dose, schedule, and histologic analysis. Current or former smokers had a response rate of 22.5%, as compared with 10.3% among patients who had never smoked cigarettes, a consistent observation through anti-PD-1 trials that needs further exploration.

The median duration of response was 12.5 months in all patients, 10.4 months in previously treated patients, and 23.3 months in previously untreated patients. Median PFS was 3.7 months for all the patients, 3.0 months for previously treated patients, and 6.0 months for previously untreated patients. Median OS was 12.0 months for all the patients, 9.3 months for previously treated patients, and 16.2 months for previously untreated patients.

Pembrolizumab was FDA approved in metastatic NSCLC expressing PD-L1, as determined by an FDA-approved test, with disease progression on or after platinum-containing chemotherapy based on data from KEYNOTE-001.

Currently pembrolizumab is being tested in different clinical settings in the context of the KEYNOTE program specially focusing on the issue of PD-L1 expression.

KEYNOTE-010 (NCT01905657) is a phase II/III trial in advanced PD-L1 positive NSCLC comparing two different doses of pembrolizumab. KEYNOTE-42 (NCT02220894) is a phase III trial comparing first line pembrolizumab (200 mg every 3 weeks) for up to 35 treatments with platinum-based doublet chemotherapy in PD-L1 positive NSCLC patients. Very similar is the KEYNOTE-24 (NCT02142738) trial that shares the same design but enrolls only PD-L1 strong positive patients. KEYNOTE-021 (NCT02039674) is a phase I/II trial in PD-L1 positive NSCLC exploring the combinations of pembrolizumab with Ipilimumab or chemotherapy.

Outside KEYNOTE program, Hoosier Cancer Research Network is conducting LUN14-179 (NCT02343952) a phase II trial of adjuvant pembrolizumab after chemo-radiotherapy for stage III NSCLC patients.

Data in SCLC patients were presented in May 2015 as preliminary results of KEYNOTE-028 (NCT02054806) an ongoing multi-cohort, phase Ib study of pembrolizumab in patients with PD-L1+ advanced solid tumors. The SCLC cohort had an ORR of 35% with durable responses (17). Another ongoing phase II trial is testing pembrolizumab in patients with extensive stage SCLC after completion of combination chemotherapy (NCT02359019).

Efficacy of agents targeting PD-L1

Atezolizumab, durvalumab and avelumab are the three main anti PD-L1 monoclonal antibodies that are being quickly developed and will soon have phase III data in different clinical settings. Until now there are no published articles of these compounds, but preliminary results have been reported in form of abstract at American Society of Clinical Oncology (ASCO) or European Society for Medical

Oncology (ESMO) meetings.

Two studies reported at the European Cancer Congress held in September 2015 in Vienna showed positive results for atezolizumab (18). In the single-arm, phase II, BIRCH study, 667 patients with advanced NSCLC and high levels of PD-L1 were treated with atezolizumab. The ORR was 19% when atezolizumab was a first-line therapy and 17% when it was a second-line or subsequent therapy. The drug seemed to work best in patients with the highest levels of PD-L1. The second study of atezolizumab, the phase II POPLAR trial, involved 287 patients with NSCLC who had already received chemotherapy. Patients were treated with either atezolizumab or docetaxel, a standard second-line treatment for NSCLC. OS was 12.6 months in patients who received atezolizumab, compared with 9.7 months in those who received docetaxel. As in the BIRCH study, atezolizumab appeared to be most effective in patients with the highest levels of PD-L1.

The FIR trial (NCT01846416), a phase II study using atezolizumab in PD-L1 positive NSCLC patients is expected to be completed in June 2016 while several phase III trials are ongoing to test different doses and settings.

Durvalumab preliminary results of a phase I trial in patients with different solid tumor types including NSCLC reported clinical benefit and durable disease control with no dose limiting toxicities or grade 3–4 toxicities (19). Objective response was seen in 23% of patients with pretreated NSCLC (12 out of 53 evaluable patients) in the phase II trial (19).

Preliminary results from an ongoing study with 346 patients with solid tumors, of whom 143 had NSCLC, used durvalumab at 10 mg/kg every 2 weeks for 1 year. The median treatment duration was 8 weeks, and activity was seen as early as 6 weeks. After finishing active therapy, ORR in NSCLC was 13% (20).

JAVELIN clinical trial program is an extensive international program exploring the use of PD-L1 inhibition with avelumab to treat multiple types of cancer. The JAVELIN clinical trial program includes a phase III open-label, multicenter trial to investigate avelumab *vs.* docetaxel in patients with stage IIIb/IV or recurrent NSCLC that has progressed after platinum-based chemotherapy (JAVELIN Lung 200).

A phase Ib in advanced NSCLC patients progressing after platinum-based chemotherapy (NCT01772004) preliminary pre-specified analyses of 184 patients with ≥3 months follow-up was performed and reported at ASCO 2015 and updated at European Cancer Conference (ECC) 2015. Objective responses were observed in 25 (13.6%) patients. Nineteen responses were ongoing at data cutoff. Stable disease was observed in 68 patients (37.0%). Median PFS was 11.6 weeks and the PFS rate at 48 weeks was 18.1%. Median OS was 8.4 months.

Safety of anti-PD-1/PD-L1 agents

A thorough review of adverse reactions reported in trials and abstracts with anti PD-1 or PD-L1 antibodies show a very similar profile of toxicity in all five compounds described in this article. Up to 80% of patients treated with immunomodulatory antibodies experienced treatment-related adverse events of any grade, while most of them were low grade reactions.

Common low grade reactions were fatigue, asthenia, fever, chills, myalgias, headaches, dyspnea, cough, decreased appetite, nausea, and constipation (13,16).

Immune-related adverse events (irAEs) were of special interest because of the presumed mechanism of action of immunomodulatory antibodies and prior experience with anti-CTLA-4 (21,22). IrAEs included pneumonitis, vitiligo, colitis, hepatitis, hypophysitis, and thyroiditis. Treatment of severe reactions consists of withdrawal of the drug and, if required, prednisone 1 to 2 mg/kg daily should be given until the patient is back at baseline and then tapered over a month (23).

Severe adverse events (grade 3 or 4) were unfrequently seen ranging from 6–30% and treatment withdrawal rates were also low.

Few treatment-related deaths have been described, but pneumonitis was involved in most of them (12,24). Notably, this side effect was more frequent in lung cancer patients (regardless histology) that tumors from other primary origins, suggesting that the toxicity profile might be related with the localization of the disease, probably due to a local inflammatory effect. No clear relationships between the occurrence of pneumonitis and dose level or treatment duration were noted (24).

Combination of these drugs with other agents my rise the frequency and severity of side effects, and needs to be prospectively investigated. For example, when combining Ipilimumab with PD-1 inhibitors like nivolumab in melanoma, drug-related adverse events of grade 3 or 4 were reported in 53% of patients compared with 18% of patients who received Ipilimumab monotherapy (25,26). Grade 3 or 4 adverse events, regardless of attribution, were observed in 72% of patients, and grade 3 or 4 treatment-related

events were noted in 53%, with the most common events being elevated levels of lipase (in 13% of patients), aspartate aminotransferase (in 13%), and alanine aminotransferase (in 11%).

Immunologic biomarkers

PD-L1 expression in cancer cells or in tumor infiltrating lymphocytes measured by immunohistochemistry (IHC) has been postulated as a potential predictive biomarker for anti PD-1/PD-L1 antibodies (27). Interpretation of its usefulness is difficult due to the different approaches that have been used with every antibody (different techniques, cut-off points and the use of archival *vs.* contemporaneous biopsy).

One of the caveats is the fact that expression of PD-L1 may dynamically change during tumor evolution, probably in response to treatments or even as one of the mechanisms for tumor immune response escape (24).

The cut-off for PD-L1 positivity, is another important factor for the interpretation of results. For example, 1% of cut-off has been used in studies with pembrolizumab and tumors were classified in three categories: negative, light positive or strong positive. Following this classification there is a reported 30% of strong positive PD-L1 NSCLC patients (15). In the studies with nivolumab, a 5% of membrane staining of tumor cells was considered as positive. About 33-48% of tumor samples were PD-L1 positive in those trials (12). In the studies with atezolizumab, PD-L1 positivity criteria included 5% of IHC staining on tumor in infiltrating lymphocytes and tumor cells. According to these criteria, 25% of NSCLC samples were positive for PD-L1 expression (28).

Further studies are required to demonstrate if PD-L1 expression by IHC correlates with a higher response rate when tumor cells are positive for staining. Median response rates oscillate from 38% in PD-L1 positive patients (ranging from 23% to 83%) to 7% (ranging from 0% to 15%) in PD-L1 negative patients depending on the trial.

KEYNOTE 001, has shown improved ORR in patients with positivity for PD-L1 expression. Results of PD-L1 expression were reported as the percentage of neoplastic cells with PD-L1 membrane staining; objective responses among all patients was 19.4%, and the median duration of response was 12.5 months. The median PFS was 3.7 months, and median OS was 12.0 months. Intriguingly a response rate of 45.2% was seen among patients with a proportion score of at least 50% and median PFS was 6.3 months while median OS was not reached. Garon *et al.* (16) reported higher response rate and longer survival for PD-L1 positive cases.

Conclusions

Lung cancer had been traditionally considered immune resistant but nivolumab and pembrolizumab approval by FDA in 2015 pave the way for a new era in which precise immune manipulation will be essential for cancer treatment. Atezolizumab, durvalumab and avelumab will join soon the immune armamentarium but they will need to find their niche in the clinical setting. Immune modulatory antibodies favorable toxicity profile in comparison to chemotherapy is a major advantage, although much needs to be better understood in order to avoid severe autoimmune related adverse events that can spoil their clinical benefits. The presence of long lasting responses holds new hopes for physicians and patients but the tools to predict what patients are going to achieve them remain in urgent need.

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Footnote

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Nivolumab (anti-PD-1, BMS-936558, ONO-4538) in patients with advanced non-small cell lung cancer

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In the era of genomic sequencing rapid strides have been made in understanding the molecular underpinnings of non-small cell lung cancer (NSCLC) and resulted in the development of highly effective therapies targeting specific molecular subsets of the disease. Response rates (RR) have increased dramatically from ~30% with cytotoxic chemotherapy to greater than 60% with biologic therapies such as erlotinib in patients with tumors harboring specific molecular alterations. However, despite the impressive activity of targeted therapy, durable responses are uncommon and tumors tend to develop resistance to treatment within months. Thus, there is a pressing need for the development of newer modalities of treatment for advanced lung cancer.

Immunotherapy offers the hope of overcoming the inherent weaknesses of conventional systemic therapies by initiating or amplifying an effective anti-tumor immune response. In recent years tremendous changes have occurred to the landscape of immunotherapy for solid tumors. Various facets of the immune system have been targeted to break immunological tolerance induced by tumors and resulted in the development of therapeutic vaccines against tumor antigens, adoptive T-cell therapies and antibodies designed to block immune checkpoints that result in T cell anergy. In the pre-checkpoint blockade era lung cancer had been considered a poorly immunogenic tumor and results of immunotherapy had been generally disappointing (1). However, targeting of cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programed death 1 (PD-1) or its ligand (PD-L1) has resulted in potentially practice-changing observations of safety coupled with impressive and durable anti-tumor activity.

A subgroup analysis of a phase I study of nivolumab in patients with NSCLC presented by Brahmer and colleagues at the 2014 annual meeting of the American Society of Clinical Oncology provides a snapshot of the potential benefits of targeting PD-1 in patients with NSCLC (2). A total of 129 patients with previously treated NSCLC were enrolled in a large expansion cohort of this study to assess the clinical activity of nivolumab at doses of 1, 3 and 10 mg/kg. Median overall survival (OS) was 9.9 months, RR was 17% and the median duration of response (DOR) was 17 months (*Table 1*). Clinical activity was seen in all patient subgroups independent of histology, number of prior lines of therapy,

Table 1 Clinical activity of nivolumab and drugs approved for treatment of recurrent NSCLC

Intervention	Response rate (%)	Overall survival (months)	Duration of response (months)	Comments	Reference
Pemetrexed	9.1	8.3	5.3	No more than one prior line of chemotherapy	(3)
Docetaxel	8.8	7.9	4.6		
Erlotinib	8.9	6.7	7.9	Patients unselected for the presence of EGFR-sensitizing mutations; 49% patients had received ≥2 prior treatments	(4)
Nivolumab	17	9.9	17	54% patients had received ≥3 prior treatments	(2)

mutational status of NSCLC and PD-L1 expression on tumor cells.

Drugs approved for treatment of recurrent NSCLC include pemetrexed, docetaxel and erlotinib. Results of clinical trials evaluating these drugs when compared with nivolumab in the setting of previously treated, advanced NSCLC show that a larger fraction of more heavily pretreated patients appear to benefit from nivolumab with higher RR, more durable responses and longer survival (*Table 1*). Additionally, Brahmer *et al*. (2) show that treatment with nivolumab is better tolerated with grade 3-4 adverse events (AE) observed in 14% patients. In comparison, the frequency of grade 3-4 neutropenia alone was 40% with docetaxel and 5% with pemetrexed and a significant proportion of patients underwent hospitalization for neutropenic fever (13% with docetaxel and 2% with pemetrexed) or for other drug-related AEs (11% with docetaxel and 6% with pemetrexed).

The clinical activity of nivolumab in patients with previously treated NSCLC also compares favorably with standard therapy for patients earlier in the disease course with untreated, inoperable, advanced or recurrent NSCLC. The phase III AVAPERL study evaluated induction therapy with four cycles of bevacizumab (7.5 mg/kg), cisplatin (75 mg/m^2) and pemetrexed (500 mg/m^2) followed by maintenance bevacizumab (7.5 mg/kg) or bevacizumab plus pemetrexed (7.5 mg/kg; 500 mg/m^2) in patients with non-squamous NSCLC who had responded to or had disease stabilization with induction therapy (5). The OS was 15.9-19.8 months from induction and 13.2-17.1 months from randomization to maintenance therapy. The 1- and 2-year OS rates were 68-72% and 34-40% respectively. The EURTAC study evaluated erlotinib for frontline treatment of EGFR-mutated NSCLC (6). Although OS was 19.3 months, the DOR was only 8.2 months. In comparison, treatment with nivolumab at a dose of 3 mg/kg in heavily pre-treated patients is associated with median OS of 14.9 months, 1- and 2-year OS of 56% and 45% respectively, and an estimated median DOR of 74 weeks (17.3 months). Thus, for those patients who respond, the durability of the response is what sets this approach apart from prior therapeutic strategies.

With the rapid development of PD-1/PD-L1 inhibitors there is a pressing need to develop predictive biomarkers to identify patients most likely to respond to treatment. The Brahmer study showed a RR of 15% in PD-L1 expressing tumors (at least 5% tumor cells showed membranous PD-L1 staining using the Dako immunohistochemistry (IHC) assay utilizing clone 28-8) and 14% in PD-L1-negative tumors. However, with a 1% cut-off for PD-L1 expression, the RR in PD-L1-positive and negative tumors was 13% and 17% respectively. This is in contrast to observations by Garon *et al*. who showed a RR of 24% in PD-L1-positive tumors (≥1% tumor PD-L1 expression with a prototype IHC assay using the 22C3 antibody) versus 8% in PD-L1-negative tumors (7). These results highlight the need for developing standardized assays and threshold criteria to determine PD-1/PD-L1 expression before using it as a selection criterion for enrollment on clinical trials evaluating anti-PD-1/PD-L1 antibodies. Since a fairly large proportion of patients with PD-L1 negative tumors also derives benefit from PD-L1 inhibition, it is imperative to understand the role of other biologic variables like the tumor microenvironment that could potentially determine sensitivity to checkpoint inhibition (8). The role of somatic mutations in determining response to checkpoint inhibitors in NSCLC also needs further evaluation. It has been observed that tumors with a high frequency of somatic mutations such as melanoma and NSCLC are more likely to respond to anti-PD-L1 therapy (9). Among patients with NSCLC, the mutational rate is higher in current or former smokers compared to never smokers and the former have been observed to have higher RR to anti-PD-L1 inhibition than the latter. It is also important to realize that not all mutations are immunologically relevant and have an ability to mediate tumor rejection. The presence of certain epitopes (rejection antigens) may play a more important role in influencing the ability of tumors to respond to checkpoint inhibitors than the mutational frequency itself.

Another important observation from the study by Brahmer *et al*. (2) is the relationship between the dose of nivolumab and OS. The longest median survival of 14.9 months and the highest 1- and 2-year survival rates of 56% and 45% respectively were noted at a dose of 3 mg/kg with an apparent reduction in median survival to 9.2 months at a dose of 10 mg/kg. Although only 59 patients were treated at a dose of 10 mg/kg compared to 37 patients at 3 mg/kg, and these cohorts were not randomized, the differences observed suggest caution when assuming a higher dose will lead to better activity with biologic agents. Higher RR and greater survival at dose levels lower than the highest dose level evaluated have been observed in trials of pembrolizumab in advanced, untreated PD-L1-expressing NSCLC (10) and nivolumab in advanced melanoma (11). These observations merit further evaluation to determine the optimal biological dose of immunotherapies such as checkpoint inhibitors.

The promising results obtained with nivolumab in advanced, previously treated NSCLC have spawned the

development of several clinical trials which have the potential to generate practice-changing data. Nivolumab has been evaluated against docetaxel in previously treated NSCLC in two phase III trials that have completed accrual (NCT01673867 and NCT01642004) with results expected soon. Nivolumab is also being evaluated against cytotoxic chemotherapy in the front-line setting in a phase III trial (NCT02041533). An ongoing phase I trial is being conducted to determine the safety and tolerability of adding nivolumab to various treatment regimens including chemotherapy, bevacizumab, ipilimumab and erlotinib in patients with advanced lung cancer (NCT01454102). Secondary endpoints of this study include determination of the objective response rate and the progression free survival rate. The addition of nivolumab to ipilimumab aims to take advantage of the phenomenon of immunologic intensification with a goal of generating deeper and more durable responses while minimizing toxicity. Ongoing phase I trials of nivolumab in combination with anti-killer immunoglobulin receptor (KIR) antibodies and lymphocyte activation gene 3 (LAG-3) inhibitors will also help in determining the benefit of immunological intensification in NSCLC (NCT01968109 and NCT01714739). Future studies need to focus on the role of nivolumab as part of multimodality therapy in the neoadjuvant and adjuvant setting for patients with early-stage or locally advanced NSCLC.

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Footnote

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Nivolumab as first line monotherapy for advanced non-small cell lung cancer: could we replace first line chemotherapy with immunotherapy?

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While conventional chemotherapy has been the cornerstone of first line treatment of advanced non-small cell lung cancer (NSCLC) historically, there is a great appeal to the concept of bypassing this potentially toxic and only modestly effective approach with molecularly targeted therapies or immunotherapies that hold the promise of greater efficacy and improved quality of life. Over the past 5-10 years, we have seen novel agents such as epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) inhibitors demonstrate striking activity and a generally favorable toxicity profile that have led these agents to be widely adopted as first line therapy ahead of conventional chemotherapy, albeit only in narrowly and molecularly defined subsets. One of the leading aspects of the excitement around immune checkpoint inhibitors such as nivolumab is that they appear to have impressive clinical activity that is not limited to a particular histologic subtype or comparatively small subpopulation, and with a very different and typically milder range of adverse effects than standard chemotherapeutic agents.

Earlier work with nivolumab has demonstrated that this agent can lead to dramatic and durable responses in a minority of patients with advanced NSCLC, as well as some other cancer types (1). This work, however, was in previously treated and sometimes very heavily pre-treated patients, in whom immunotherapy was not competitive with established therapies. While the prolonged responses seen in a minority of patients in this early work suggest the possibility of obviating more toxic and potentially less effective chemotherapy, we have yet to see direct comparisons of the efficacy of nivolumab or other immune checkpoint inhibitors in head to head trials with established chemotherapy standards. Clinical trials that have completed enrollment already directly compared second-line docetaxel to nivolumab in patients with squamous (2) or non-squamous (3) advanced NSCLC, though we don't have results at this time. But to have an immune checkpoint inhibitor displace initial treatment with cytotoxic chemotherapy as the cornerstone of initial therapy for the majority of patients with advanced NSCLC, we would need to see comparable or superior efficacy with the improvement in toxicity profile that these agents promise.

The abstract by Drs. Gettinger and colleagues (4) represents a promising initial effort to assess the potential utility of nivolumab as monotherapy preceding conventional chemotherapy in a relatively broad clinical population that includes patients with either squamous or non-squamous NSCLC, while also seeking to determine whether patients with tumor PD-L1 expression above a 5% threshold using their particular test (DAKO kit, clone 28-8) is associated with significantly greater probability of clinical benefit with nivolumab than PD-L1 negative tumors (4). The study, with a primary endpoint of assessing safety and tolerability of nivolumab as first line therapy, reported at ASCO on the first 20 patients, who split fairly evenly between squamous and adenocarcinoma NSCLC histologies (ten adenocarcinoma, nine squamous, one other); patients with an EGFR mutation or ALK rearrangement were excluded. Patients had been followed a median of 66 weeks.

At the time of study analysis, 15 of the 20 (75%) had

discontinued therapy, 11 of whom (55%) for disease progression, two (10%) for adverse events (AEs), and one additional patient each (5%) for an unrelated AE or per patient request. Six patients (30%) had an objective response, including two (10%) with a complete response; among these patients, responses were ongoing in four (20%). Another seven patients (35%) demonstrated stable disease as their best response, with progressive disease in the remaining seven patients (35%). There were no clear differences based on tumor histology, with objective responses seen in two of nine (22%) patients with squamous NSCLC, compared with four of 11 (36%) patients with non-squamous NSCLC.

The biomarker of PD-L1 expression was explored in 17 patients, of whom 10 (59%) were designated as PD-L1 positive, of whom five (50%) were responders, and seven (41%) as PD-L1 negative, among whom there were no responders (0%). However, the progression free survival (PFS) at 24-week and 1-year survival were relatively comparable between PD-L1 positive and negative patients (70% *vs*. 57% and 80% *vs*. 71%, respectively).

As has been characteristic of research with immune checkpoint inhibitors thus far, tolerability was overall quite favorable. Specifically, while 17 of 20 patients (85%) experienced at least one treatment-related AE, these were only grade 1 or 2 in 13 of these 17 patients (76%). The two patients who terminated treatment due to serious AEs of elevated transaminases or cardiac failure [1 (5%) each] both recovered after discontinuation of treatment. There were no cases of pneumonitis observed.

What conclusions should be drawn from this early work? A preliminary report on 20 patients cannot overturn the overwhelming preponderance of data on the survival benefit of conventional chemotherapy accumulated over hundreds of trials run over several decades. What this limited report offers is a clear proof of principle that a minority of patients can benefit profoundly from nivolumab, experiencing dramatic and potentially prolonged responses to immunotherapy with good tolerability.

The key issue in interpreting the significance of this research effort is to place it into proper context rather than view it with "irrational exuberance" of envisioning a chemotherapy-free world for most lung cancer patients. At this point, we must recognize that the response rate is very comparable with but not clearly superior to that of standard chemotherapy regimens in the first line setting, and that having 10% of patients discontinue treatment due to prohibitive AEs, with another 10% coming off due to unrelated AEs or clinical judgment does not represent an overwhelming signal of dramatically improved efficacy or tolerability for nivolumab in this setting. While a subset of patients experience marked benefits, tumor histology does not provide predictive guidance about which patients are most likely to benefit. The leading candidate as a predictive biomarker, PD-L1, has no remote consensus for adoption in terms of lab-based technique or threshold for designating patients as positive or negative; accordingly, correlations of outcomes of PD-L1 expression with clinical outcomes of patients treated with various immune checkpoint inhibitors have been most notable for their consistency only in demonstrating a higher response rate in patients considered as PD-L1 expressing, but this marker is neither necessary nor sufficient for observing an objective response or prolonged survival with immune checkpoint inhibitors (5).

Taken together, these data offer a glimpse of a possible future in which nivolumab or another immune checkpoint inhibitor could displace standard chemotherapy as first line therapy for some patients with advanced NSCLC. Before that happens, however, we will need to be able to reliably identify the subset of patients most likely to benefit from immunotherapy and see large-scale trials that directly compare nivolumab or another immune checkpoint inhibitor directly against conventional platinum-based doublet chemotherapy with a prospectively defined improvement in efficacy and/or tolerability.

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Footnote

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Nivolumab for refractory metastatic squamous non-small-cell lung cancer: fulfilling an unmet need

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Lung cancer is the leading cause of cancer death in the United States and worldwide (1,2). Approximately 85% of cases involve non-small-cell lung cancer (NSCLC), of which 30% will be squamous cell histology. Of those squamous cell lung cancers diagnosed yearly, it is estimated that more than 50% are metastatic at diagnosis. Until recently, the recommended treatment for good performance status patients with metastatic squamous lung cancer consisted of first-line platinum doublet chemotherapy followed, upon disease progression, by second-line single agent chemotherapy (1). Median overall survival from initial diagnosis of metastatic squamous lung cancer in patients who receive first-line platinum doublet chemotherapy ranges from 8-11 months (3,4). Therefore, effective new therapies are desperately needed. Building upon durable objective responses to the anti-programmed death-1 (anti-PD-1) antibody, nivolumab, reported in phase I studies, Rizvi *et al*. have recently published the results of a single-arm phase II trial of single-agent nivolumab in pretreated metastatic squamous lung cancer (5). In conjunction with headline results from a randomized phase III trial in second-line metastatic squamous lung cancer confirming improved OS for nivolumab compared with docetaxel, these data have led to the FDA approval of nivolumab for the treatment of metastatic squamous lung cancer after prior platinum-based chemotherapy (6).

Immunotherapy, an approach to modulate a patient's own immune system thus destroying cancer cells, has demonstrated preliminary efficacy and safety in several phase I studies in advanced NSCLC (7,8). The programmed death-1 (PD-1)/programmed death ligand-1 (PDL-1) pathway has been identified as a co-inhibitory pathway that may be activated by cancer cells to protect against host immune system elimination (8). The use of nivolumab, a fully human IgG4 PD-1 immune checkpoint inhibitor antibody, to 'turn off' this inhibition, and upregulate immunosurveillance therefore destroying cancer cells, is an area of active study in advanced squamous cell lung cancer.

In "Activity and safety of nivolumab, an anti-PD-1 immune checkpoint inhibitor, for patients with advanced, refractory squamous NSCLC (CheckMate 063): a phase II, single arm trial" by Rizvi *et al*., the investigators enrolled 117 patients with stage IIIB or IV squamous cell NSCLC from 27 international sites between November 2012 and July 2013. These patients had all received at least two prior therapies, and 76% were less than three months from completion of their previous regimen suggesting that this was not a population with slowly progressive or indolent cancer. The dose of nivolumab (3 mg/kg every 2 weeks until disease progression or unacceptable side effects) was not modified throughout the trial, and treatment after progression was permitted if a patient had investigator-assessed clinical benefit and was otherwise tolerating therapy. The primary endpoint of the study was objective response rate (ORR) by RECIST 1.1 and was confirmed by an independent radiology review committee. The ORR in this study was 14.5% and responses occurred independently of age, sex, baseline performance status, region, ethnicity and number of previous treatments. Exploratory endpoints included evaluation of exposure-

response relationships, characterize pharmacokinetics, immunogenicity, safety and tolerability of nivolumab; as well as calculations of progression-free survival and overall survival of all treated patients. The authors also evaluated the association between the proportion of patients with an ORR and PD-L1 expression in all patients. Median time to response was 3.3 months and median duration of stable disease was 6 months; median duration of response was not reached at the time of report. Median progression-free survival was 1.9 months, and median overall survival was 8.2 months. Pretreatment archival tumor samples from 88% of participants were assessed for PD-L1 expression; 25 of these 76 patients (33%) were found to have PD-L1 positive tumors (≥5% expression). The authors report that responses occurred more frequently in PD-L1 positive tumors however it should be noted that the difference in ORR between PD-L1 positive versus negative was not statistically significant. Treatment-related adverse events were reported by 74% of patients, and were most commonly fatigue, decreased appetite and nausea. Grade 3-4 toxicities, most commonly fatigue, pneumonitis and diarrhea, occurred in 17% of patients. Treatment-related adverse events led to study discontinuation for 14 of 117 (12%) patients, most commonly pneumonitis (4%) and fatigue (2%). Two deaths were attributed by the investigator to nivolumab; one patient died of hypoxic pneumonia 28 days after the last dose of nivolumab, and another patient passed away from an ischemic stroke 41 days after the last dose of nivolumab. The authors concluded that nivolumab monotherapy provides clinically meaningful activity and an acceptable safety profile for patients with advanced refractory squamous NSCLC.

Discussion

Rizvi *et al*. demonstrated that nivolumab is a safe therapeutic option for advanced squamous NSCLC. Indeed, this study was cited by the FDA as establishing a promising safety profile that, in combination with interim analysis results of the Phase III CheckMate-017, has led to approval of nivolumab for use in advanced squamous NSCLC with progression on or after platinum-based chemotherapy (6). With the exception of ECOG performance status (all patients on this study were ECOG 0-1) the patient cohort in this study was a realistic reflection of advanced squamous lung cancer patients, all patients were heavily pretreated and shared characteristics indicative of an aggressive biology. The dosing of nivolumab, obtained from prior phase I studies, demonstrated an ORR not significantly different to that reported in other studies of nivolumab in advanced NSCLC (7). Using RECIST 1.1 criteria and an independent radiology review ensured that the data would be generalizable and in general there was good concordance between the assessments.

When discussing the results of single-arm, phase II studies in advanced cancer an important question is what constitutes "success". While the ORR in this study was more than acceptable for the patient population (one might expect an ORR of 5-10% with generic single agent chemotherapy in this patient group), what is most attractive is the likely durability of response. In a previous phase I study of nivolumab in 129 patients with heavily pretreated NSCLC the median duration of response was 17 months (9). This duration of response to a systemic therapy is unprecedented in the treatment of advanced NSCLC. While long-term follow up on the current study is not yet mature, one might expect a similar prolonged benefit for those patients who do respond. Predicting which patients will respond to immune checkpoint inhibition has proven challenging. The authors note that numerically at least more patients with PD-L1 positive tumors had an objective response however this difference was not statistically significant. At present PD-L1 expression on tumors cannot be recommended as a rigorous biomarker for the selection of NSCLC patients for treatment with nivolumab.

Overall, nivolumab appeared to be well-tolerated, with the rate of adverse events lower than in similar studies evaluating cytotoxic chemotherapy, in addition the two patient deaths on this study appear to have been multifactorial. Further studies are needed to identify those patients at risk for potential immunologic adverse events, so they can be evaluated and treated appropriately. As has been found in other studies, progression-free survival is not a particularly relevant endpoint in immunotherapy trials given the tendency for these agents to benefit a subgroup of patients who derive prolonged benefit i.e., the tail on the survival curve. As a primary endpoint, overall survival of patients treated with nivolumab is being actively assessed in other studies (including in the first-line setting), and the results of these studies are being actively analyzed at this time. This will assist us in more fully understanding where nivolumab belongs in the treatment paradigm of NSCLC. Overall, CheckMate 063 identifies a promising role for nivolumab in the role of fulfilling the unmet need of new treatment options in progressive metastatic squamous NSCLC.

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Footnote

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Pembrolizumab as first-line treatment for non-small cell lung cancer—a game changer?

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Non-small cell lung cancer (NSCLC) (80–85% of all lung cancers) continues to be one of the major causes of cancer related deaths around the world (1). For the vast majority of patients with advanced or metastatic disease (40–50% of all patients at time of diagnosis) platinum-based chemotherapy remains the only potential treatment and has led to significantly improved survival outcomes with a "plateau" of about 10–11 months median survival (2).

Subsequently, significant advances have been made with the introduction of pemetrexed, especially against the non-squamous cell subtype. The addition of this agent led to a further improvement in survival to 12–13 months (3) and up to 14 months with the introduction of maintenance therapy (4). Nonetheless, even with these therapies, the majority of patients with NSCLC do not attain prolonged disease control (5).

The development of molecularly targeted therapies (small molecules and monoclonal antibodies) has, however, significantly improved outcomes in the metastatic setting for NSCLC patients harbouring activated oncogenes such as epidermal growth factor receptor (*EGFR*) and translocated anaplastic lymphoma kinase (*ALK*). In addition, immune checkpoint inhibitors have also dramatically changed the therapeutic landscape of NSCLC. In particular, monoclonal antibodies targeting the programmed death-1 (PD-1)/PD ligand 1 (PD-L1) pathway have emerged as powerful new therapeutic tools in several clinical trials (6).

On the basis of these early efficacy signals, a series of randomized phase II and III studies have been conducted to evaluate the activity of PD-1/PD-L1 inhibitors in the second-line setting and beyond in NSCLC (7).

In the CheckMate-017 (NCT01642004) and CheckMate-057 (NCT01673867) studies, for example, the PD-1 inhibitor nivolumab produced significant improvements in overall survival (OS) compared with docetaxel in patients with previously treated, squamous and non-squamous NSCLC, respectively (8,9). Similarly, the PD-1 inhibitor pembrolizumab improved OS compared with docetaxel among patients with previously treated NSCLC whose tumours expressed PD-L1 (10).

Notably, however, in these second-line studies (e.g., CheckMate-017 and CheckMate-057), the control arms received single-agent docetaxel (75 mg/m^2), which set a low bar to clear [historical overall response rate (ORR) approximately 7%] (11). By contrast, in the first-line setting, PD-1/PD-L1 inhibitors will need to trump platinum-based doublet chemotherapy, which has been associated with much higher ORRs (25% to 35%) (2,3). For PD-1/PD-L1 inhibitors to succeed in this setting, a biomarker enrichment strategy might clearly be necessary.

Recently, the activity and improved tolerability of PD-1/PD-L1 inhibitors in NSCLC have provoked an increasing interest in moving these agents to the first-line setting. Gettinger *et al.* (12) reported findings from a cohort of 52 patients with advanced NSCLC treated with first-line nivolumab monotherapy (CheckMate-012, NCT01454102, a multi-arm phase IB study, N=412). Importantly, PD-L1 testing was not used to prospectively select patients for study entry, but mandatory pre-

treatment biopsies were required to evaluate PD-L1 expression retrospectively. Confirmed responses were observed in 23% of patients, including complete responses in four patients (8%). Median progression-free survival (PFS) for nivolumab was 3.6 months, but the median duration of response was not reached (range, 4.2 to 25.8 months). Thus, as with other studies of PD-1/PD-L1 inhibitors, there was a suggestion of durable clinical responses. Indeed, the median OS in this patient population was noteworthy at 19.4 months. From this study the authors concluded that first-line nivolumab monotherapy demonstrated a tolerable safety profile and durable responses in NSCLC patients.

In another study Rizvi *et al*. (13), who reported a separate first-line cohort of CheckMate-012, evaluated nivolumab in combination with platinum-based doublet chemotherapy for first-line treatment of advanced or metastatic NSCLC (CheckMate-012, NCT01454102). Patients (N=56) received nivolumab plus platinum-based doublet chemotherapy concurrently every 3 weeks for four cycles followed by nivolumab alone until progression or unacceptable toxicity. Regimens were nivolumab (10 mg/kg) plus gemcitabine-cisplatin (squamous-cell) or pemetrexed-cisplatin (non-squamous) or nivolumab (5 or 10 mg/kg) plus paclitaxel-carboplatin (all histologies). The primary objective was to assess safety and tolerability. Secondary objectives included ORR and 24-week PFS rate; exploratory objectives included OS and response by tumour PD-L1 expression.

In this study confirmed responses were seen in 33% to 47% of patients across all chemotherapy arms. Notably, these response rates were not substantially different from those expected with platinum-based doublet chemotherapy alone, but 2-year OS rates offered a hint of additional activity, particularly in the nivolumab (5 mg/kg) plus carboplatin-paclitaxel arm.

Despite intriguing signals in certain subsets of CheckMate-012, it has to be highlighted that this was a non-randomized study and it was conducted with a relatively low number of patients. Moreover, the degree of benefit in both studies appeared to be modest. For instance, in the KEYNOTE-001 (NCT 01295827) study (14) pembrolizumab was associated with an ORR of 24.8% among treatment-naive patients, but the response rate increased to 50% among treatment-naive patients who were PD-L1 positive (proportion score >50%).

On the basis of these observations, two randomized phase III studies, CheckMate-026 and KEYNOTE-024, had been launched in PD-L1-positive NSCLC patients comparing platinum-based chemotherapy *vs*. nivolumab or pembrolizumab, respectively.

Results from the KEYNOTE-026 (NCT02142738) phase III trial (N=305) of pembrolizumab (200 mg, day 1, every 3 weeks up to 35 cycles or until documented progressive disease) *vs*. platinum-based chemotherapy as first-line therapy for patients with metastatic NSCLC that expresses PD-L1 (≥50%) have been published most recently (15). Pemetrexed maintenance therapy was permitted for patients with non-squamous histologies and patients harbouring sensitizing *EGFR* mutations and/or *ALK* translocations were not enrolled. In addition, patients randomized to the control arm had the option of crossing over to pembrolizumab upon disease progression. The trial's primary endpoint was PFS and the secondary endpoint was OS. Pembrolizumab significantly improved PFS (10.3 versus 6.0 months, P<0.001, HR =0.50) compared with chemotherapy. In addition, the estimated rate of OS at 6 months was 80.2% in the pembrolizumab group versus 72.4% in the chemotherapy group (P=0.005). Furthermore, ORR was higher in the pembrolizumab group than in the chemotherapy group (44.8% versus 28.8%).

Since pembrolizumab was superior compared to chemotherapy for both, the primary endpoint of PFS and the secondary endpoint of OS, an independent data monitoring committee (IDMC) has recommended that the trial had to be stopped, and that patients receiving chemotherapy in this trial had be offered the opportunity to receive pembrolizumab.

Based on the data presented so far an approval of pembrolizumab as first-line treatment for PD-L1-positive NSCLC patients is expected in the near future. This would then move nivolumab in the third line of treatment since the drug is only approved in the EU/US for NSCLC patients with prior chemotherapy treatment (16). However, it should be noted that only 23–28% of all patients with advanced NSCLC have a high level of PD-L1 expression, which is defined as membranous PD-L1 expression on at least 50% of tumour cells, regardless of the staining intensity (10,15).

By contrast, results from the CheckMate-026 (NCT02041533) phase III, open-label, randomized study of nivolumab as monotherapy *vs*. investigator's choice chemotherapy in patients with advanced NSCLC have

been detailed in parallel (17). Patients enrolled in that trial had received no prior systemic treatment for advanced or metastatic NSCLC and were tested positive for PD-L1 expression (cut-off threshold: ≥5%). The trial randomized 541 patients to receive either nivolumab (3 mg/kg every 2 weeks) or investigator's choice chemotherapy in squamous patients (gemcitabine with cisplatin/gemcitabine with carboplatin/paclitaxel with carboplatin) and non-squamous patients (pemetrexed with cisplatin/pemetrexed with carboplatin) until disease progression, unacceptable toxicity, or completion of six cycles. The primary endpoint was PFS as assessed by the independent radiology review committee (IRRC). Secondary endpoint was OS. Again, patients harbouring sensitizing EGFR mutations and/or ALK translocations were not enrolled, and an optional cross-over was allowed.

The trial did not meet the primary endpoint of PFS in untreated, advanced NSCLC patients whose tumours expressed PD-L1 of at least 5%, and full results will be published in greater detail soon (17).

Meanwhile, data were published from a new analysis comparing nivolumab plus ipilimumab *vs.* nivolumab alone in previously untreated NSCLC patients (CheckMate-227, NCT02477826). Presented at the American Society of Clinical Oncology meeting this year (press release) the results showed that 57% of the combo patients responded, compared with the 28% in the nivolumab arm, and the response rates were higher in patients with higher levels of PD-L1 (18). Final results are expected to be published end of 2020.

In terms of future clinical studies, well-conducted randomized trials will be crucial in determining the role of PD-1/PD-L1 inhibitors in the first-line setting. Indeed, more than 11 such trials are currently ongoing (*Table 1*).

In general, the following two major trial designs/groups can be identified: (I) PD-1/PD-L1 inhibitor monotherapy *vs.* platinum-based doublet chemotherapy in biomarker-selected (i.e., PD-L1-positive) patients, and (II) PD-1/PD-L1 inhibitors plus platinum-based doublet chemotherapy *vs.* platinum-based doublet chemotherapy alone in a general population with NSCLC (not biomarker-selected).

In addition, the combination of two immune checkpoint inhibitors [e.g., durvalumab (anti-PD-L1) and tremelimumab (anti-CTLA-4)] are also under evaluation and may provide an OS benefit for NSCLC patients. In this regard, data from two ongoing clinical trials (MYSTIC, NCT02453282 and NEPTUNE, NCT02542293) are eagerly awaited (*Table 1*).

This question remains what the more promising strategy would be? Unfortunately, current data are still too preliminary to draw firm conclusions, but it is conceivable that both approaches may be used for future clinical trials. In addition, it can be assumed that PD-1/PD-L1 inhibition alone may be sufficient in small, biomarker-defined subsets of patients (e.g., PD-L1-positive tumours), whereas combinations may be necessary for unselected or PD-L1-negative patient populations (19).

With the recent regulatory approvals of nivolumab (Opdivo®, Bristol-Myers Squibb, New York, USA) and pembrolizumab (Keytruda®, Merck & Co., Kenilworth, USA) in the US and Europe, first-line studies of PD-1/PD-L1 inhibitory monoclonal antibodies may now have to struggle with cross-over designs, making assessments of OS more difficult. As a result, a majority of ongoing phase III trials evaluating PD-1/PD-L1 inhibitors in the front-line setting still rely on PFS as a primary end point (*Table 1*).

In addition, it will be mandatory to incorporate tissue analyses into future trials of PD-1/PD-L1 inhibitors to facilitate insight into predictive biomarkers of response and resistance. This might be most relevant in the first-line setting because such patients generally have a number of other therapeutic options (e.g., treatment with TKIs, chemotherapy, monoclonal antibodies). Despite an emphasis on PD-L1 testing in ongoing trials, it is clear that the role of PD-L1 expression alone is far for being clear since there is still a number of pitfalls (e.g., heterogeneity of expression, threshold differences in PD-L1 assays, lack of a gold standard for PD-L1 positivity) (20).

In summary, despite these encouraging results it is unlikely that immune checkpoint inhibitors will completely replace platinum-based chemotherapy as first-line treatment for NSCLC, but the development of better predictive biomarkers may allow oncologists to identify particular subsets that are most likely to benefit from PD-1/PD-L1 pathway blockade in first-line, either alone or in combination with chemotherapy or other checkpoint inhibitors which are currently in clinical development. In addition, it remains to be seen whether immune checkpoint inhibitors may also be of benefit for first-line treatment of NSCLC patients harbouring sensitizing *EGFR* mutations and/or *ALK* translocations.

Table 1 Ongoing first-line phase III studies with immune checkpoint inhibitors for advanced and/or metastatic NSCLC

Drug	Trial number	N	Design	Endpoints	PD-L1 status	Completion date
Pembrolizumab	NCT02775435 (KEYNOTE-047)	560	Carboplatin/nab-paclitaxel (or paclitaxel) with or without pembrolizumab (squamous cell)	OS, PFS (primary); ORR (secondary)	NS	August 2019
	NCT02578680 (KEYNOTE-189)	570	Platinum/pemetrexed with or without pembrolizumab (non-squamous)	PFS (primary); OS, ORR (secondary)	NS	March 2019
Avelumab	NCT02576574 (JAVELIN Lung 100)	420	Avelumab *vs*. platinum-based doublet chemotherapy (all histologies)	PFS (primary); OS (secondary)	Positive only	June 2023
Durvalumab	NCT02453282 (MYSTIC)	1,092	Durvalumab with or without tremelimumab *vs*. SOC (non-squamous)	PFS, OS (primary); PFS for durvalumab alone (secondary)	NS	June 2018
	NCT02542293 (NEPTUNE)	800	Durvalumab/tremelimumab *vs*. SOC (non-squamous)	OS (primary); OS in PD-L1-negative patients, PFS (secondary)	Required retrospectively	October 2018
Nivolumab	NCT02477826 (CheckMate-227)	1,980	Nivolumab *vs*. nivolumab plus ipilimumab *vs*. platinum-doublet chemotherapy (non-squamous)	PFS, OS (primary); ORR (secondary)	Testing required	December 2020
Atezolizumab	NCT02409342 (IMpower 110)	570	Atezolizumab *vs*. platinum/ pemetrexed (non-squamous)	PFS (primary); OS (secondary)	Positive only	March 2019
	NCT02657434 (IMpower 132)	680	Atezolizumab plus platinum/ pemetrexed *vs*. platinum/ pemetrexed (non-squamous)	PFS (primary); OS (secondary)	Testing required	May 2019
	NCT02366143 (IMpower 150)	1,200	Atezolizumab plus carboplatin/ paclitaxel with or without bevacizumab (non-squamous)	PFS (primary); ORR, OS (secondary)	NS	November 2022
	NCT02409355 (IMpower 111)	NS	Atezolizumab *vs*. platinum/ gemcitabine (squamous cell)	PFS (primary); ORR, OS (secondary)	Positive only	September 2017
	NCT02367794 (IMpower 131)	1,200	Atezolizumab plus carboplatin/ nab-paclitaxel (or paclitaxel) *vs*. carboplatin/nab-paclitaxel (or paclitaxel (squamous cell)	PFS (primary); OS (secondary)	NS	February 2023
	NCT02367781 (IMpower 130)	550	Atezolizumab plus carboplatin/ nab-paclitaxel *vs*. carboplatin/ nab-paclitaxel (non-squamous)	PFS (primary); ORR, OS (secondary)	NS	January 2019

NSCLC, non-small cell lung cancer; PD-L1, PD ligand 1; PFS, progression-free survival; OS, overall survival; ORR, overall response rate; NS, not stated; SOC, standard of care.

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Integrating the molecular background of targeted therapy and immunotherapy in lung cancer: a way to explore the impact of mutational landscape on tumor immunogenicity

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Abstract: The results of randomized clinical trials employing immune checkpoint inhibitors for pre-treated advanced non-small-cell lung cancer (NSCLC) have recently revolutionised the standard available option for this disease setting. Nevertheless, the validation of reliable predictive biomarkers, able to define that proportion of patients most likely to benefit from immunotherapy, represents a crucial and still unsolved issue. This intensive research aimed at selecting potentially predictive biomarkers for immunotherapy is developed together with a wide range of analyses investigating the molecular profiling of lung cancer, leading to the spontaneous question of how these two parallel aspects of the same disease may coexist and influence one another. The potential impact of the mutational landscape of lung cancer on tumor immunogenicity (in both oncogene-addicted and molecularly unselected disease) will be explored and discussed in this review in order to begin to answer the unsolved questions.

Keywords: Immunotherapy; lung cancer; mutations; neoantigens; immunogenicity

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Introduction

In recent years, technological advances in the study of biological background of tumors provided the proof-of-principle that non-small-cell lung cancer (NSCLC) is represented by a group of molecularly heterogeneous diseases. Several genetic mutations have been identified and validated as oncogenic drivers, able to determine the development and maintenance of specific subclasses of NSCLC (1).

The thrilling discovery is that several mutations are 'actionable', or rather targetable with specific drugs, radically transformed the care and prognostic perspectives of lung adenocarcinoma patients. The era of targeted therapy in lung cancer broke through with the discovery of driver mutations in the epidermal growth factor receptor (EGFR) (2,3). Several randomized clinical trials conducted in NSCLC carrying activating mutations of EGFR clearly demonstrated that tyrosine kinase inhibitors largely improve prognosis, disease control, symptoms and quality of life when compared to traditional platinum-based

chemotherapy (4,5). Other potentially targetable driver mutations have been identified in lung adenocarcinoma, including HER2, MET and fusion oncogenes involving anaplastic lymphoma kinase (ALK), ROS1 and RET (1,6).

The introduction of tumor genotyping into therapeutic decision-making, the discovery of new potential targets and the technological advances in multiplexed genotyping platforms, led to development of several large-scale screening programs to determine the true frequency of oncogenic drivers (7-9). The final aim is to provide practical routine molecular profiling techniques able to collect reliable information to guide treatment of patients and simplify studies with targeted agents (10).

Recently, the therapeutic opportunities of lung cancer patients further expanded with the introduction of immunotherapy. The great excitement among scientists, physicians and patients after the presentation of successful results in treating subsets of cancer patients quickly led to the onset of pressing questions regarding what parameters may predict response. The intensive research aimed to identify potentially predictive biomarkers for immunotherapy is developed together with the above-described investigations about the molecular profiling of lung cancer, leading to the spontaneous question of how these two parallel aspects of the same disease may coexist and influence one another.

Immunotherapy and predictive biomarkers

The results of randomized clinical trials employing immune checkpoint inhibitors for pre-treated advanced NSCLC have recently revolutionised the standard available option for this disease setting, with significant advances for squamous histology and good perspectives for non-squamous (11,12). Nevertheless, while nivolumab demonstrated a significant improvement in terms of survival for squamous histology regardless of the immunohistochemistry expression of the programmed-death ligand-1 (PD-L1), the benefit of receiving this antibody in comparison with docetaxel seems to be higher in those patients with high tumoral PD-L1 expression in the case of non-squamous NSCLC (regardless of the cut-off, 1%, 5% or 10%) (11,12). The different impact of the candidate predictive biomarker PD-L1 according to histology is still debatable. In this regard, although the benefit of nivolumab seems to be restricted to those patients with high tumoral PD-L1 expression in the case of non-squamous NSCLC, the same correlation has not been observed in squamous histology. Thus, the retrospective evaluation of PD-L1 expression in archival samples does not lead to definitive conclusions. Moreover, in the pivotal trial of pembrolizumab in NSCLC, although patients with squamous histology represented only a small proportion, the predictive effect of PD-L1 seems to be confirmed using contemporaneous samples (13). Therefore, the hypothesis that the impact of a rich cohort of coexisting mutations (as in the squamous subtype) may overcome the predictive power of PD-L1 must be validated.

The results of the Checkmate 057 are coherent with those of the randomized phase II POPLAR trial employing the anti-PD-L1 atezolizumab for all the histologies of NSCLC, although the immunoscore for biomarker positivity comprise both the expression on tumoral and tumor-infiltrating immune cells (14). Although the overall results about the predictive role of PD-L1 are convincing, still unsolved issues are represented by the determination of the best cut-off expression level and the different analytic techniques adopted across different trials. In this regard, advanced NSCLCs receiving pembrolizumab (an anti-PD-1 immune checkpoint inhibitor) are significantly much more likely to benefit from this drug if PD-L1 is strongly expressed upon tumor cells (>50%) (13).

Currently, the complexity of factors triggering the immune response to efficiently recognize and neutralize a specific antigen can not be easily simplified by the direct pharmacodynamics of an antibody binding PD-L1. As recently demonstrated, other immune mediators are potentially involved in atezolizumab-driven immune responses and only partially mediated by PD-L1 overexpression (15). In addition, patients with low PD-L1 expression may respond to an anti-PD-L1 antibody as well, underscoring the complexity of biological mechanisms supporting the immune response. PD-L1 may probably be considered just one of the predictive factors for immunotherapy and recent data suggest that the combination of other markers of immune cell infiltration (such as CD10 and CD20) and their ratios may have a prognostic (and maybe predictive) implication (16).

The expression of PD-L1 (regardless of the method), and all the other biomarkers of immune microenvironment are significantly affected by analytical and reproducibility limitations with important implication for clinical practice. Thus, the reported practical difficulties in interpreting the results of trials according to PD-L1 expression, strongly call for the identification and validation of biologically relevant and reliable biomarkers, determined with reproducible and harmonized assay procedures (17).

Impact of mutational landscape on response to immunotherapy

The identification and validation of those factors able to determine tumor immunogenicity represents a major challenge for research in the immunotherapy field. The immunogenicity of a tumor depends on its antigenicity and a series of immunomodulatory factors produced both by tumor and host cells in the tumor microenvironment (18).

Tumor-specific antigens can be classified into two main categories: tumor-associated self-antigens (such as cancer-testis and differentiation antigens) and antigens derived from tumor specific mutant proteins [also called neoantigens or mutation-associated-neo-antigens (MANA)]. While T-cell reactivity against self-antigens is usually weak and characterized by a low avidity binding, neoantigens are fully human specific, and are therefore theoretically expected to induce a stronger immune response without toxicity against healthy tissues. The production of neoantigens is induced by a mutational event that may involve antigen expression as well as its processing and presentation to immune cells (19).

The finding that immune cell populations in tumor infiltrates may affect responsivity to checkpoint inhibitors highlights the necessity to understand which antigens can induce an effective immune response against the tumor.

Some preliminary studies suggested that tumors with a high load of somatic mutations are more likely to respond to immunotherapy through the presentation of neoepitopes that may behave as neoantigens (20-22). To test this hypothesis, Snyder *et al.* performed whole-exome sequencing of tumor samples from melanoma patients treated with the anti-CTLA-4 specific antibodies ipilimumab and pembrolizumab. As expected, the high load of somatic mutations correlated with response to therapy in most patients, but surprisingly not in all. Computational analysis demonstrated that specific mutation-derived neoepitopes were shared by those patients responding to immunotherapy, defining a signature able to predict long-term clinical benefit from checkpoint blockade (23). In this regard, the quality of mutations, more than the quantity, may have the strongest predictive value (24). The identification of those mutations producing immunogenic neoantigens, able to trigger an effective immune response, is essential to the understanding and manipulation of T-cell response against cancer.

Available data support the fact that T-cell adaptive immune response might be preferentially directed towards a specific subset of mutant sequences, facilitating the bioinformatic identification of possible neoantigens for therapeutic targeting (25). Yadav *et al.* developed, in the context of a murine tumor, an innovative approach that combines whole-exome and transcriptome sequencing analysis with mass spectrometry to identify neo-epitopes. Vaccination of mice confirmed the reliability of this approach, virtually applicable in any cancer cell type, with each predicted immunogenic peptide yielding therapeutically active T-cell responses. Interestingly, the identified neoantigens usually derived from proteins not directly related to tumorigenesis, enhancing the significant role of passenger mutations in the determination of cancer immunogenicity (26). Another pivotal study used genomic and bioinformatic approaches to rapidly and accurately identify tumor-specific mutant proteins, useful not only as targets of checkpoint inhibitors, but also as components of major histocompatibility complex (MHC) tetramers that can be used to identify tumor-specific T-cells as biomarkers of successful immune responses against cancer (27). In this regard, Kreiter *et al.* proposed a complex approach by integrating technological advances in the field of next-generation sequencing, computational immunology and synthetic genomics to explore the neoantigen repertoire in order to identify those that are most immunogenic (according to their expression level and MHC class II-binding capacity). Vaccination with synthetic poly-neoepitope messenger RNA vaccines, produced against these carefully selected neoantigens, induces tumor rejection of established growing tumors in mice models (28).

NSCLCs, particularly those related to the chronic exposure to carcinogens in cigarette smoke, are usually characterized by a high mutational burden, representing a biologically rationale target for immunotherapy approach (29). In this regard, the pivotal study of Rizvi *et al.* explored the potential influence of the NSCLC mutational landscape in determining sensitivity to PD-1 blockade (with pembrolizumab) (30). Whole exome sequencing, conducted in two independent cohorts, demonstrated that patients with high nonsynonymous mutation burden, compared with those with low mutation burden, experienced improved objective response rate (63% *vs.* 0%), progression-free survival (14.5 *vs.* 3.7 months) and durable clinical benefit (73% *vs.* 13%) from pembrolizumab. Efficacy was also correlated with molecular smoking signature, higher neoantigen burden and DNA repair pathways mutations (30).

Several studies reported that only a tiny fraction of neoantigens is predicted to bind to MHC molecules,

becoming effective targets of endogenous T-cell response. Nevertheless, from a purely probabilistic point of view, tumors with a high number of mutation-associated neoantigens are more likely to produce effective epitopes, stimulating the antitumor immune system reaction. This hypothesis supports the correlation between the high mutational load and the response rate observed with anti-CTLA-4 in melanoma and anti-PD-1 in lung cancer (23,30).

According to this hypothesis, even tumors with mismatch-repair deficiency (MRD) could represent potentially strongly immunogenic disease. In fact, MRD colorectal cancers have 10 or 100 times as many mutations as mismatch repair-proficient (MRP) cancers (31). Moreover, they are characterized by a prominent lymphocytes infiltrate supporting an effective immunogenic value (32,33). To validate this hypothesis, a phase II trial evaluating the clinical activity of pembrolizumab has been conducted in progressive metastatic carcinoma patients with or without MRD (34). Patients with MRD colorectal cancer demonstrated a clinical benefit of immune checkpoint blockade with pembrolizumab compared to those with MRP cancers, both in terms of immune-related response rate (40% *vs.* 0%) and of immune-related progression-free survival (78% *vs.* 11%). A statistically significant prolongation of median progression-free survival and overall survival favouring the cohort with MRD tumors was also reported. According to the available evidence, the high mutational load was associated with prolonged progression-free survival (P=0.02). In this regard, with whole-exome sequencing analysis MRD tumors presented a mean of 1,782 mutations per tumor as compared with 73 in MRP tumors (P=0.007) (34).

Immunogenicity in oncogene-addicted disease

As previously discussed, several genetic mutations have been identified and validated as oncogenic drivers in NSCLC (1). This finding, in the context of immunotherapy research, implies intriguing questions regarding the interaction and mutual influence of the two pathways, particularly in terms of response to treatment (both with tyrosine kinase inhibitors and immunotherapeutic agents).

Besides the above-described limitations in terms of both analysis and interpretation, PD-L1 seems to be differentially expressed according to the molecular phenotype of tumors. In this regard, a recent analysis assessed PD-1/PD-L1 expression in NSCLC patients harboring EGFR mutations, ALK translocations or KRAS mutations (35). Whereas PD-1 positivity was significantly associated with active smoking status (P=0.02) and with the presence of KRAS mutations (P=0.006), PD-L1 positivity correlated to adenocarcinoma histological subtype (P=0.005) and EGFR mutations (P=0.001). PD-L1 positivity was also associated with improved benefit from gefitinib and erlotinib in terms of response rate (P=0.01), time to progression (P<0.0001) and overall survival (P=0.09). Interestingly, median PD-L1 levels were 5 times higher in ALK translocated tumors compared with triple negative, although the association was not statistically significant (35).

A growing body of evidence suggests that oncogenes may indirectly influence tumor microenvironment, regulating the release of ligands and cytokines (36). EGFR represents one of the most commonly mutated oncogenes in NSCLC patients (37). Preclinical studies conducted in murine melanoma models demonstrated that the activation of EGFR might suppress the immune response against cancer (38). Based on these findings, a pivotal study analysed the immune microenvironment and the immune-related pathways in EGFR-driven mouse lung tumors (39). A correlation between EGFR activation and a composed signature of immunosuppression (manifested by the upregulation of PD-1, PD-L1, CTLA-4 and several tumor-promoting inflammatory cytokines) was reported. This role of the EGFR pathway was independent of its traditional activity in cell proliferation and survival, suggesting an active involvement of EGFR as a modulator of tumor microenvironment. Concerning pharmacological inhibition, the tyrosine kinase inhibitors targeting EGFR reduced PD-L1 expression with a positive impact on mice survival. On the other hand, PD-1 antibody blockade improved the survival of mice with EGFR-driven adenocarcinoma by both targeting tumor cells and inducing the activity of T-cells, modulating the expression of immuno-regulatory cytokines. Globally considered, these findings suggest that concurrent inhibition of PD-1 and EGFR pathways may represent a rational and promising approach for EGFR-addicted NSCLC (39).

Our group performed next-generation sequencing to assess the mutational status of a series of EGFR-mutant advanced lung cancers receiving first line gefitinib. The results of our study suggested that the presence of additional coexisting mutations significantly decreases the expected benefit of tyrosine-kinase inhibitors. This finding has a biological rationale. While the presence of a high mutation burden may predict benefit from immunotherapy in unselected lung cancer in the context of an oncogene-

addicted disease, additional coexisting mutations suggest an underlying molecular heterogeneity, leading to by-passing of the main oncogenic stimulus (40).

In contrast to EGFR-activating mutations, KRAS mutations are usually detected in smokers and associated with poor prognosis and no benefit from tyrosine kinase inhibitors and adjuvant chemotherapy (1,41). An integrative analysis of genomic, transcriptomic and proteomic data was recently performed in both chemotherapy-naïve and heavily pre-treated KRAS-mutant lung adenocarcinoma (42). Three biologically distinct subsets of KRAS-mutant cancer were identified by co-occurring genetic alterations in STK11/LKB1 (KL subgroup), TP53 (KP subgroup) and CDKN2A/B inactivation with low TTF1 expression (KC subgroup). Regarding immune system engagement, KP tumors were characterized by an intense inflammatory response with enhanced expression of several costimulatory and coinhibitory factors, including PD-L1. In contrast, KL KRAS-mutant lung adenocarcinoma appeared almost immune-inert. Despite the similar exposure to smoking, KP lung adenocarcinoma showed a higher global mutation rate compared with KL tumors and this finding may contribute to explain the reported differences in terms of immunogenicity between these two subgroups of KRAS-mutant cancer (42).

Only preliminary evidence is available about the immune related aspects of the ALK fusion oncogene that seems to possess an intrinsic immunogenicity value inducing T-cell responses and humoral immunity (43).

Globally considered, the results obtained in the available studies exploring the immunogenicity of oncogene-addicted lung cancer are still preliminary and debatable. A prospective validation in the context of a larger population is mandatory in order to definitively validate the role of major lung cancer oncogenes as reliable parameters to predict the awaited effect derived from PD-1/PD-L1 inhibition.

Conclusions

Increasing evidence is available to support the role of neoantigens in inducing and maintaining anti-tumor responses to immunotherapeutic agents. In this regard, the burden of random mutations arising during normal DNA replication of non-cancerous stem cells—which lead to the development of cancer—is not perhaps as 'unlucky' as it might at first seem, at least as far as the implications for immunotherapy are concerned (44).

If the quantity of neoantigens statistically correlates with the probability of response to immunotherapy, strategies aimed to enhance the production of tumoral neoantigens may theoretically be combined with immunotherapy to improve the expected benefit. In this regard, one of the most promising approaches is radiotherapy. Radiation therapy targeted selectively to the tumor acts as an *in situ* tumor vaccine by inducing release of antigens during cancer cell death in association with pro-inflammatory factors able to trigger the innate immune system to activate tumor-specific T-cells. If successful, not only does it result in the rejection of the irradiated tumor, but also in the rejection of the systemic disease (a phenomenon known as abscopal effect) (45).

Nevertheless, pivotal trials demonstrated that the quality of neoantigens probably matters more than the global mutation burden. Technological advances in genomics and bioinformatics have provided promising tools to efficiently select the strongest immunogenic neoantigens from the broad spectrum of somatic mutations in a tumor. The aim is a 'reverse immunology' approach going from theory (computational epitope prediction) to practice (*in vitro* validation).

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Footnote

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Moving towards a customized approach for drug development: lessons from clinical trials with immune checkpoint inhibitors in lung cancer

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Abstract: Lung cancer has recently been discovered to be an immunological targetable disease, on the basis of the exciting results of the randomized trials with immune checkpoint inhibitors. Nevertheless, the survival benefit appears to not be entirely captured by the usual outcome measures, thus requiring a deep reflection about the appropriateness of the traditional statistical methodologies in this context. The intrinsic biological differences existing both in terms of mechanism of action and kinetic between immunotherapy and chemotherapy or targeted therapy, impact on patients' outcome, requiring a global revolution in the way to design clinical studies with the ideal aim to evolve towards trials carefully 'customized' on the basis of the investigational drug, the specific disease and the biological background. The exciting data recently obtained with immune checkpoint inhibitors, offer an ideal context and background to explore the major questions and future perspectives about the development of immunotherapeutic agents. In this regard, the choice of adequate endpoints, the use of modified statistical methods and the potential introduction of predictive biomarkers for immunotherapy clinical trials, will be discuss in this review in order to provide practical and rationale suggestions aimed to improve the existing model for cancer immunotherapy investigation.

Keywords: Immunotherapy; lung; cancer; trial; design

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Introduction

For a long time, the disheartening results obtained in early and advanced investigation with immunotherapeutic agents were justified by the employment of inactive or only marginally active agents and by the incomplete understanding of human tumor immunology. In lung cancer in particular, these disappointing results translated into the common belief that lung cancer represented a non-immunogenic disease, where immunotherapy could achieve only a marginal success.

In recent years, immunologic and clinical science significantly evolved, refusing this preliminary uncorrected axiom and providing a new portrait of lung cancer as an immunological targetable disease. In this regard,

the recent thrilling discovery that the high mutational burden, characterizing in particular the not oncogene-driven non-small cell lung cancer (NSCLC), is associated with improved clinical benefit from antibody targeting programmed cell death-1 (PD-1), identifies NSCLC as an ideal target for immunotherapy, similarly to melanoma (1). Considering the now-validated immunogenic potential of lung cancer, the only marginal benefit observed in past years with immunotherapy should probably be attributed to the employment of inadequate statistical methodologies, unable to completely capture the real benefit deriving from the use of immunotherapeutic agents (2).

The intrinsic biological differences existing in term of mechanism of action and kinetic between immunotherapy and chemotherapy or biological therapy, indicates that a new drug development paradigm is needed. Immunotherapeutic agents act as a continuum of biological events, that starts early with immune system activation and that procrastinates until the potential ideal obtainment of a usually delayed clinical benefit.

These biological differences imply that the clinical benefit of immunotherapy, when present, manifests as an overall survival (OS) benefit, replacing the main role achieved by the progression-free survival (PFS) in the era of targeted agents. Thus, the global way to design clinical trials should be theoretically revolutionized in order to adapt to the survival behavior of patients treated with immunotherapeutic agents (graphically represented by the new shape of Kaplan-Meier curves where long survivors and delayed benefit strongly matter).

All major issues and future perspectives regarding the development of immunotherapeutic agents in lung cancer, including choice of adequate endpoints, use of modified statistical methods and introduction of predictive biomarkers in immunotherapy clinical trials, will be explored and argued in this review.

Analysis of end-points in the context of randomized trials

The traditional end-point for advanced NSCLC from both the clinical trial design and the regulatory agencies perspectives has been OS, at least unless molecularly featured patients' subsets were identified, and specific targeted agents demonstrated to change the prognosis of such particular (and relatively rare) diseases (3). Indeed, with the exception of those drugs approved according to the expression of a specific biomarker (i.e., EGFR, ALK) (4-7), a series of targeted agents failed to be approved by regulatory agencies on the basis of PFS alone, even if positive and/or clinically meaningful (8). The most represented drug-class in this regard refers to antiangiogenics, although bevacizumab and, recently, ramucirumab and nintedanib, have been worldwide approved in the context of unselected or histology-restricted patients (8-11).

The mathematical model which medical oncologist were used to, traditionally attributed to PFS a larger benefit in favour of the investigational drug in comparison with OS. Although controversial and with different interpretations, PFS was introduced as a reasonable end-point for new drugs to be approved from regulatory agencies in a series of solid tumors, including lung cancer. Indeed, in the context of successful trials, experimental drugs were expected to significantly improve PFS with a larger difference in comparison with the standard treatment (usually preceded by an improvement in response rates), than what expected for OS. That was the scenario for many targeted agents for unselected patients, with many trials reproducing the results of the AVAIL trial, where bevacizumab significantly improved PFS, without any benefit in OS (12). As already reported in a series of analysis, the presence of a correlation, its strength and power, between end-points may significantly vary according to drug types (so depending by their mechanism of action) and disease setting (13). As a general rule, in many scenarios a significant correlation between PFS and OS was demonstrated, indicating the larger benefit in PFS did translate in smaller advantages in OS (14). To simplify, we can speculate by assuming that generally with chemotherapy, and with a series of targeted agents, the ratio between the hazard ratio (HR) of PFS and the HR of OS has been since now almost always less the 1, although few (but important) exceptions (15). This paradigm failed to be replicated in the context of randomized trials with immune checkpoint inhibitors for advanced NSCLC (*Table 1*).

Nivolumab was recently tested for pretreated advanced NSCLC in the phase III fashion in comparison with the standard of care docetaxel; two twin trials (Checkmate 017 and 057) were prospectively designed to demonstrated the superiority of nivolumab over docetaxel according to histology. These data represent the first release of phase III data with immune-checkpoint inhibitors for NSCLC in the context of unselected patients for (any) biomarker (16,17,19).

With regard to the Checkmate 017, the study was designed to determine a HR of 0.74 at the final analysis (power 90%), with a difference in median OS of 2.5 (7-9.5)

Table 1 Characteristics of randomized clinical trials comparing immune checkpoint inhibitors with chemotherapy in NSCLC

Trial	Phase	Disease subtype	Agent	Treatment arms	Pts	Primary end-point	HR (95% CI) (P value)	Secondary end-point	HR (95% CI) (P value)
CHECKMATE 017 (16)	III	SQLC	Nivolumab	Nivolumab (3 mg/kg) every 2 ws *vs.* docetaxel (75 mg/m^2) every 3 ws	135 *vs.* 137	OS	0.59 (0.44-0.79) (P<0.001)	PFS	0.62 (0.47-0.81) (P<0.001)
CHECKMATE 057 (17)	III	Non-SQLC	Nivolumab	Nivolumab (3 mg/kg) every 2 ws *vs.* docetaxel (75 mg/m^2) every 3 ws	290 *vs.* 292	OS	0.73 (0.59-0.89) (P=0.0015)	PFS	0.92 (0.77-1.11) (P<0.3932)
POPLAR (18)	II	NSCLC	Atezolizumab	Atezolizumab (1,200 mg) every 3 ws *vs.* docetaxel (75 mg/m^2) every 3 ws	144 *vs.* 143	OS	0.77 (0.55-1.06) (P=0.11)	PFS	0.98 (0.75-1.2)* (P=n.r.)

*, intervals derived from ASCO 2015 presentation. Pts, patients; HR, hazard ratio; CI, confidence interval; OS, overall survival; PFS, progression-free survival; SQLC, squamous lung cancer; NSCLC, non-small cell lung cancer; ws, weeks; n.r., not reported.

months, in favour of nivolumab for patients candidate to receive second-line docetaxel (randomization 1:1), affected by squamous NSCLC. At a (estimated) median follow-up of 15 months (272 enrolled patients, 73% of deaths, with censored patients almost exclusively in the right part of the Kaplan-Meier curve), nivolumab significantly improved OS in comparison with docetaxel, with a HR of 0.59 [95% confidence interval (CI), 0.44-0.79; P<0.001]. Median OS was significantly longer for patients receiving the experimental drug (9.2 months) in comparison with docetaxel (6 months); of particular note, 1-year OS resulted to be almost doubled with nivolumab (42% *vs.* 24%). According to what reported in the paper, no difference in third line treatment are present. Progression-free-survival resulted to be improved for patients receiving nivolumab with a HR of 0.62 (95% CI, 0.47-0.81; P<0.001), although the difference in medians is actually too small to be considered clinically relevant (from 2.8 to 3.5 months). The study was originally designed with overall response rate (ORR) as co-primary end-point, assuming to detect a response rate difference of 25% (power 90%); ORR resulted to be higher with nivolumab (P=0.008), although less than what expected (20% *vs.* 9%). To summarize, just looking at the HR, we can conclude that in the case of nivolumab for squamous NSCLC, the ratio between the HRs of PFS and OS results to be higher than 1 (1.05).

In the Checkmate 057 (designed to compare survival in patients with non-squamous NSCLC), at a (estimated) median follow-up of 17 months, nivolumab significantly improved OS in comparison with docetaxel, with a HR of 0.73 (95% CI, 0.59-0.89; P=0.0015). Median OS was significantly longer for patients receiving the experimental drug (12.2 months) in comparison with docetaxel (9.4 months), with a 1-year OS of 51% for nivolumab *vs.* 39% for docetaxel. No significant difference in PFS was determined, with a HR of 0.92 (95% CI, 0.77-1.11). Similarly, a small difference in ORR in the whole populations was found (19% *vs.* 12%). Thus, in the context of non-squamous NSCLC, the ratio between the HRs of PFS and OS results to be 1.26.

The interim analysis of the randomized phase II trial POPLAR, at a (estimated) median follow-up of 11 months, showed that atezolizumab did not significantly improve OS (HR, 0.77; 95% CI, 0.55-1.06) nor PFS (HR, 0.98) in comparison with docetaxel for second line NSCLC (regardless of histology) (18). In this case, the ratio between the HRs of PFS and OS results to be 1.27.

Thus, on the basis of the randomized trials to date available comparing these drugs *vs.* chemotherapy, as

reported before, immune checkpoint inhibitors seem to alter the current paradigm assessing that the benefit in PFS is larger than that obtained in OS, given their PFS/OS ratio ranging from 1.05 to 1.27.

The potential role of biomarkers in clinical trials with immune checkpoint inhibitors

The potential predictive role of immune-related biomarkers represents a crucial aspect in recent clinical investigation with immune checkpoint inhibitors, in order to identify that proportion of patients most likely to benefit from immunotherapy, leading to an optimized therapeutic index for these agents.

The detection of PD-L1 (which can be constitutively or inducible expressed on either the surface of tumor cells or upon cancer-infiltrating T-cells) as a potential predictive biomarker, represents one of the most investigated strategy in clinical trials (20). Several early-phase trials in NSCLC have suggested that PD-L1 expression, usually detected by immunohistochemistry (IHC) on tumor cells, may increase the likelihood of response to anti-PD-1 or anti-PD-L1 antibodies. In this regard, we recently conducted a sensitivity analysis of trials in the context of advanced melanoma, NSCLC and genitourinary cancers demonstrating that nivolumab, pembrolizumab and atezolizumab provide a significant differential effect in terms of activity according to PD-L1 expression on tumor cells (21).

Taking into account the randomized trials with anti-PD-1 pathway inhibitors in the context of NSCLC patients, the potential predictive value of PD-L1 expression did not constantly emerged. Indeed, the Checkmate 017 trial, demonstrated a benefit in terms of objective response, PFS and OS with nivolumab regardless of tumor cells IHC PD-L1 status, across all the pre-specified expression levels subgroups (1%, 5%, and 10%) (16,19). On the contrary, in the Checkmate 057 trial, the tumor PD-L1 positivity, starting at the lowest expression level (1%), emerged as a significantly predictive biomarker of benefit for nivolumab in terms of both activity and efficacy (17). Moreover, the differential effect of the biomarker was independent from the PD-L1 cut-offs adopted (1%, 5% and 10%). Similar results are reported by Spira *et al*. in the POPLAR trial, where atezolizumab provided a significant differential effect in objective response, PFS and OS according to the IHC PD-L1 expression on tumor cells and/or tumor-infiltrating immune cells (18). At this regard, others studies demonstrate that, besides the PD-L1 expression by tumor cells, the localization of PD-L1 expression on immune cells infiltrating the tumor may impact on the employment of PD-L1 as a potential predictor of clinical response (22,23). The different cut-offs and methods adopted for the detection and the quantification of PD-L1, such as the type of anti-PD-L1 antibody, the staining techniques, the criteria for classify a 'positive' tumor and the samples used for the assay, do not allow to easily compare the results of studies and to deeply speculate about the reliance and consistency between them.

Despite these limitations, the PD-L1 expression on tumor cells or infiltrating immune cells may potentially represent a reasonable candidate biomarker for the selection of patients affected by NSCLC, in order to optimize the treatment strategy with immune checkpoint antibodies. Thus, the analytical validation of this potential biomarker according to a universally shared positivity cut-off is warranted. In addition, the clinical validation of PD-L1 expression as a predictor of efficacy requires further phase III trials stratified according to PD-L1 status and prospective analysis in large cohorts of patients with PD-L1 positive or negative NSCLC.

Recent findings suggest that several other immune regulatory pathways may be involved in the response to PD-1/PD-L1 pathway blockade, such as PD-L2, another PD-1 ligand, PD-1 expression, tumor-infiltrating lymphocytes (TILs) and immunogenic neo-antigens, these last associated with a high mutation burden, that play an important role in immunogenicity (22,24,25). At this regard, the results reported by Rizvi and collegues analyzing a population of NSCLC patients treated with pembrolizumab, demonstrated that a high mutation burden is strongly associated with clinical benefit from PD-1 blockade. Efficacy also correlated with rate of nonsynonymous mutations, alteration in DNA repair pathway, molecular smoking signature and high neoantigens burden (1).

Perspectives for trial design with immune checkpoint inhibitors

As previously described, OS represents the traditional end-point for cancer clinical trials from both the clinical trial design and the regulatory agencies perspectives. To avoid the indiscriminate approval of drugs on the basis of only small survival benefit, restrictive criteria have been defined in order to establish the minimum incremental

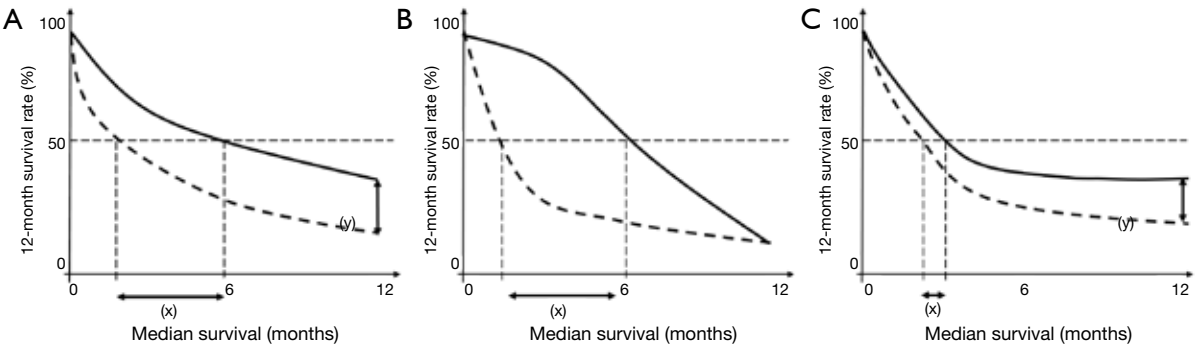

Figure 1 Typical survival curves (Kaplan-Meier model) observed in clinical trials. A detailed description of the different panels (panel A, B and C) is reported in the text. (x), difference in median survival; (y), 12-month difference in survival rate.

improvement over standard therapy that would define a clinically meaningful outcome (26).

The results of the Checkmate 017 and 057 randomized trials (and the POPLAR as well, even if a phase II) clearly indicate that, at least for the two immune check-point inhibitors in the more advance phase of development (nivolumab and atezolizumab), the common belief that large benefits in PFS should be obtained in order to determine a significant (despite smaller) benefit in OS, should be abandoned. In addition, the results of these trials in terms of medians strongly call for a deep reflection upon which outcome measures should be chosen to better intercept the effect of an experimental drug.

Traditionally, trials are designed and sized upon the basis of expected differences in terms of medians or proportional reduction in the risk of the chosen events (i.e., the Cox model, if applicable). Such modeling may be significantly affected by the 'shape' of the Kaplan-Meier curves, so that these two methods may not easily intercept the benefit of a given drug or treatment. In this regard, it must be addressed that the analysis of the reduction of the proportional risk of a certain end-point (represented by the HR) is just one of the way to look to differences in continuous variables, and the introduction of immunological agents for cancer therapy may represent a crucial point to revolution the traditional way to design clinical trials and to evolve towards trials 'customized' upon the drug-type, the disease setting and (hopefully) the biological background.

Figure 1 displays three typical survival curves (according to the Kaplan-Meier model) which each individual oncologist and regulatory agencies may have to deal with when interpreting trial results in the context of a specific clinical scenario:

- Panel A shows the results of a theoretical clinical trial whereas the effect of the experimental drug (continuous line) is captured by either the difference in median survival (x) and the HR (i.e., the reduction in the risk of event is maintained proportional during the observation). In addition, the difference captured by the median, is later maintained when performing a landmark analysis of patients event-free at 12 months (y). In this case, we can imagine that the drug starts to work early (curves initiate to separate at the beginning of the study), and continues to be active along the treatment is administered in the same way. Thus, for the clinical trial in panel A, both median and HR would have been ideal when prospectively designed the study;
- In panel B, the results of a clinical study whereas the effect of the drug starts very early (curves dramatically separate immediately). The large benefit in favour of the experimental drug is captured entirely by the medians (x), but, as long as patients relapse in both arms, the differences disappear, and no difference at the 12-month landmark analysis is determined. This is the typical curve of the PFS of targeted agents for the case of oncogene-addicted disease (i.e., EGFR mutant or ALK-rearranged); the drug has a dramatic efficacy earlier (as mirrored by the strong activity in terms of response rates), but resistance occurs and the vast majority of patients (at 12 months in this case) has required a further line of treatment. In such case, to design and power the study on the basis of a difference in medians may allow to provide a trial with a relatively small sample; the drug-efficacy may be captured by using HR as well, although the curves-crossing at a certain time may render this measure not entirely appropriate;

- Panel C shows what to date randomized trials with immune checkpoint inhibitors are displaying in terms of PFS and OS. The benefit of the experimental drug in comparison with the standard is detected later in the study. Medians do not capture the effect of the drug (x), while the landmark analysis may be the best tool to magnify the (y). This is particularly true for immunotherapy in general (i.e., cytokines, ipilimumab and immune checkpoint inhibitors for melanoma), where the effect showed with a landmark analysis is replicated at 18- and 24-month in both lung and melanoma (27). As for panel B, HR may be used, although the reduction in the risk of the event in the first part of the curve is not proportional.

Actually, we cannot consider these tools similar or one substitute of the other, not only for the operative characteristics as explained before. Indeed, each one of them (medians, HR, landmark analysis) may mirror the efficacy of the drug in different contexts. As recently reported, a different key may be adopted to explain deeply this effect: median and HR may replicate the average benefit in the context of the whole sample randomized in the study, while the landmark analysis may identify long-terms survivors, i.e., those (few) patients really benefiting from the drug in terms of survival (as the drug which works is able to select patients on the basis of prognosis) (28).

This intriguing interpretation has implication for translational research: as reported before, the effect of drug dramatically working from the beginning (for example: tyrosine kinase inhibitors for EGFR mutant NSCLC patients), which may be captured by medians (or HR), may be easily monitored at early phases of the trial we are conducting. That allows to change hypotheses to better increase the success rate of the study and to provide more reliable data for clinical practice as well. That represents the principle of adaptive designs for the early phases, which may easily been applied in such context with this kind of drugs. This fascinating approach (i.e., adaptive design) is hard to be applied for drugs working lately (*Figure 1C*), and in general, in those context whereas the cancer cells are not addicted to a specific pathway which is significantly killed by a given inhibitor.

With these perspectives, the magnification of the benefit to be captured by more appropriately designs for trials and disease settings whereas immune checkpoints inhibitors are employed represents a challenge, given that: (I) the correlation with traditional intermediate end-points (such as PFS) does not work, as reported in precedent chapters; (II) the expression of PD-L1 demonstrate to significantly interact with the treatment effect in non-squamous disease, while no interaction is revealed for squamous histology; (III) biomarkers currently investigated (i.e., PD-L1, mutational load, lymphocytes infiltrate) suffer from analytical and reproducibility issues.

With regard to the latest issue, it seems clear for example that patients with high levels of PD-L1 derive more benefit from these inhibitors, as it seems particularly true when the cut-off is higher for pembrolizumab (29), but the effect is seen in negative patients as well, and it could not entirely considered negligible. According to the results of the Checkmate 057, a patient displaying a 4% PD-L1 expression can potential benefit from nivolumab if considering the 1% threshold as well as not if considering the 5% cut-off (17). Thus, the methodology and the cut-off represents a crucial issue, without forgetting that the expression of such factors depends upon where it is measured (cancer cells, immune cells, etc.) and when it is measured according to the exposure to other treatments (chemotherapy, radiotherapy, etc.). The immune reaction to cancer cells is a dynamic process and patients will be required to be monitored for that, although we currently do not have clear, reproducible and reliable methods to do that, although we have hints that other serological parameters may potentially have a role in this regard (22). Thus, unless a reliable biomarker (whatever it will be, PD-L1, immune cells infiltrate, mutational load, other) is identified, the current ways to improve the success rate of trials (basket and umbrella designs) do not seem to be appropriate for immune-oncology. In this regard, we recently understood that the simple molecular abnormality (for example BRAF mutation V600E) does not imply the efficacy of the specific inhibitor (i.e., vemurafenib), and that the disease context (and the histology) significantly matters, thus not confirming the absolute reliance of basket trials (30). The significant role of histology is highlighted by the results of the Checkmate 017 and 057 as well, whereas PD-L1 results to be predictive only for non-squamous histology.

From the pure trial perspective, the curve's shape we face with immunotherapy (*Figure 1C*), requires appropriate designs, given it may deviate from proportional hazards, and the delayed separation may imply a loss of the statistical power (low number of events), with significant implication for the sample size (31). For such reasons, alternative statistical methods should be considered to compute the required number of events for delayed separated curves (simulation, numerical integration) and quantification of

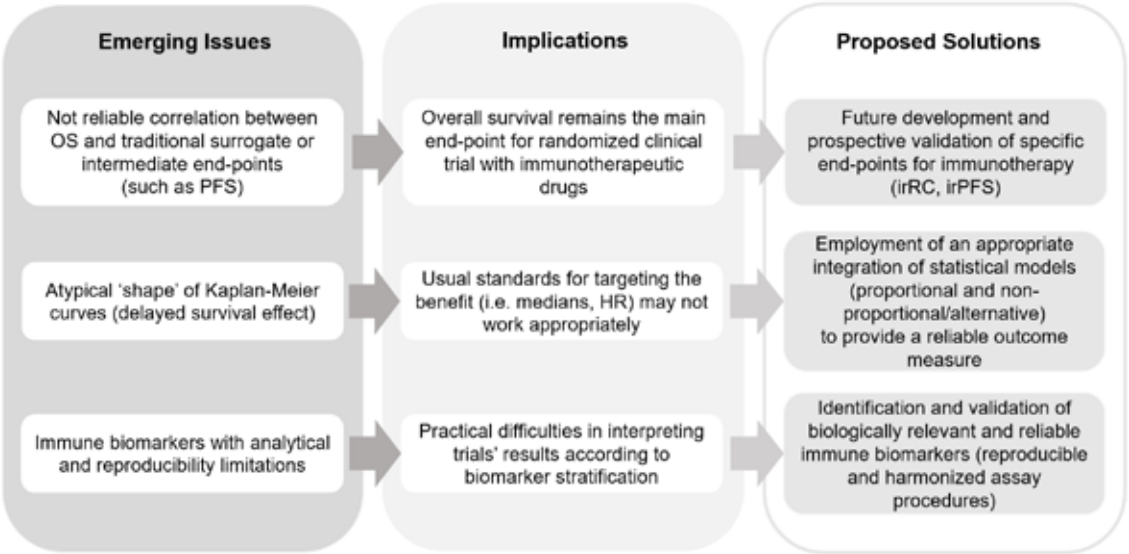

Figure 2 Perspectives for trial design with immune checkpoint inhibitors: emerging issues and proposed solutions. PFS, progression-free survival; OS, overall survival; irRC, immune-related response criteria; irPFS, immune-related progression-free survival.

the delayed effect (2). In this regard, for the reasons already described, none of the usual surrogate end-point (i.e., PFS, responses, etc.) can be considered useful for immune-oncology, and survival must remain the primary end-point of randomized trials.

In order to better capture the efficacy of the newest upcoming immune checkpoint inhibitors, a non-proportional hazard model should be adopted, and outcome measure assessing events as landmark analysis should be incorporated for the trial' hypotheses and the sample size quantification. In this regard, landmark analysis may consider absolute differences at a certain time-point (i.e., absolute difference between patients event-free at 1 year) or relative differences at that time-point (i.e., event-based risk- or odds-ratio). Given the peculiar shape of the curve (*Figure 1C*), whereas HR works only after the median, trials would better designed if the primary end-point would be targeted with both the proportional assumption (i.e., the target HR) and the relative (or absolute) landmark analysis. That would allow increasing the chances to intercept the eventual benefit of the investigated immunotherapy, and to avoid the risk to consider negative a study whereas the drugs is able to identify a patients' subgroup who significantly benefit from the newest treatment. A graphical summary of the emerging issues of immunotherapy clinical trials design with the possible solutions is proposed in *Figure 2*.

Certainly, intermediate end-point is anyway needed, in order to accelerate the process to introduce a good drug in the context of the available treatments for a given disease. Thus, cancer research is going forward to develop specific end-points for immunotherapy. In this regard, immune-related criteria for both response and adverse events are to date under validation, and immune-related PFS is going forward to be prospectively considered for future trials with these drugs.

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Footnote

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New strategies in immunotherapy for non-small cell lung cancer

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Abstract: Treatment for the most common form of cancer (lung cancer) has historically involved use of cytotoxic chemotherapy. With the advent of mutation analysis, more therapies beyond traditional cytotoxics have been discovered. Most recently, the use of immunotherapy has entered the treatment arsenal of non-small cell lung cancer (NSCLC). This review aims to summarize the current and future use of immunotherapy in the treatment of NSCLC.

Keywords: Non-small cell lung cancer (NSCLC); immunotherapy; vaccine

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Introduction

Lung cancer is the leading cause of cancer related death worldwide (1). Cytotoxic chemotherapy can improve survival, but responses are often short-lived (2). Targeted therapies result in high response rates for patients with certain alterations, such as *EGFR* mutations or *EML4-ALK* translocations, but these are found only in a minority of patients and resistance inevitably develops (3,4). Clearly, novel therapeutic approaches are urgently needed.

Utilizing the immune system to fight cancer is an attractive goal, promising the possibility of long term disease control without some of the toxicities of more traditional therapies. In order to grow, cancer must evade immune detection. In lung cancer and many other tumors, T-cell tolerance to tumor antigens is a major barrier to effective immunotherapy, and initial studies of non-specific immune stimulation with interleukin-2 and interferon-alpha showed minimal activity (5,6). Therapeutic checkpoint inhibition or activation of co-stimulatory molecules has the potential to combat this tolerance and allow anti-tumor immune responses. Recent trials with these agents have shown promising results and have renewed excitement in the field of immune therapy for lung cancer. In this article, we will review the results of recently reported clinical trials of immunotherapy agents, and we will discuss ongoing and upcoming research in this field.

Vaccine therapy

Cancers often express antigens not found on normal cells. Many clinical trials have studied the efficacy of vaccines against these antigens. Two large trials of vaccine therapy in non-small cell lung cancer (NSCLC), the Stimulating Targeted Antigenic Response to (START) NSCLC trial and the MAGRIT, a double-blind, randomized, placebo-controlled Phase III study to assess the efficacy of the recMAGE-A3 + AS15 cancer immunotherapeutic as adjuvant therapy in patients with resected MAGE-A3-positive non-small cell lung cancer (MAGRIT) trial, have recently been reported.

The START trial enrolled 1,513 patients with stage III NSCLC who had completed chemoradiation. Patients without progressive disease were then randomized to receive either tecemotide (L-BLP25), a vaccine against

Table 1 Completed NSCLC immunotherapy trials

Immunotherapy	Target	Setting	Patient number	Results
Ipilimumab	CTLA-4	Phase II	204	ir-PFS 5.7 months for phased ipilimumab + chemotherapy *vs*. 4.6 months for placebo + chemotherapy (HR 0.72; P=0.05); ir-PFS 5.5 months for concurrent ipilimumab + chemotherapy *vs*. placebo + chemotherapy (HR 0.81; P=0.13) (9)
Nivolumab	PD-1	Phase III; Checkmate 017	272	OS 9.2 months for nivolumab *vs*. 6 months for docetaxel (P<0.001) (10)
Pembrolizumab	PD-1	Phase I; KEYNOTE-001	495; (Training-182); (Validation-313)	ORR 19.4%; MDR 12.5 months; MDOS 12 months (11)
MPDL3280A	PD-L1	Phase I	53; (37 evaluable)	ORR 24%; 24-week PFS 48% (12)
MEDI4736 (durvalumab)	PD-L1	Phase I	13	3 PR with two additional responses not meeting PR per irRC (13)

NSCLC, non-small cell lung cancer; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; HR, hazard ratio; ir-PFS, immune-related progression-free survival; irRC, immune-related response criteria; MDOS, median duration of overall survival; MDR, median duration of response; ORR, overall response rate; OS, overall survival; PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand of protein 1; PFS, progression-free survival; PR, partial response.

MUC1, or placebo, and the primary endpoint of the study was overall survival (OS). There was no significant difference in survival between the two arms (median OS 25.6 months with tecemotide *vs*. 22.3 months with placebo), though there was a benefit for the vaccine in patients who received concurrent rather than sequential chemotherapy (30.8 *vs*. 20.6 months, P=0.0175) (7). Unfortunately, these promising results were not confirmed in other studies, and development of this compound has been halted.

In the MAGRIT trial, patients with surgically resected stage IB-IIIA NSCLC were randomized to receive placebo or a vaccine to MAGE-A3. Eligible patients were required to have MAGE-A3 positive tumors. A total of 2,272 patients were randomized and treated. Treatment was well tolerated, but results were disappointing as the trial showed no significant improvement in disease-free survival (60.5 *vs*. 57.9 months, P=0.7379) (8).

With negative results from these two large trials, enthusiasm for vaccine-based therapy for NSCLC has waned, and focus has shifted to treatment with checkpoint inhibitors and other immunotherapy agents.

Checkpoint inhibition

A complex interaction of both inhibition and stimulation of the immune system exists to allow the appropriate destruction of pathogens and abnormal cells while preventing overstimulation that could lead to destruction of healthy cells (autoimmunity). This network of inhibitory and stimulatory signals offers multiple potential targets. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) are co-inhibitory factors ("checkpoints") that have been the focus of much research (*Table 1*).

CTLA-4 inhibitors

CTLA-4 is expressed on the surface of T-cells. It inhibits T-cell activation and decreases immune response (14,15). Inhibition of this protein with therapeutic antibodies leads to increased activation of T-cells and anti-tumor responses in some patients. Ipilimumab is an anti-CTLA-4 antibody approved for the treatment of metastatic melanoma (16). Its activity is under investigation for NSCLC.

Preliminary signs of efficacy in NSCLC were seen in a phase II study of ipilimumab in combination with chemotherapy. Patients with previously untreated metastatic NSCLC were randomly assigned to one of three groups: carboplatin/paclitaxel, carboplatin/paclitaxel with concurrent ipilimumab, or carboplatin/paclitaxel with sequential ipilimumab (9). Results were very promising with a statistically significant improvement in progression-free survival (PFS) in the sequential arm (carboplatin and paclitaxel for two cycles followed by addition of ipilimumab with chemotherapy for four more cycles) compared to the chemotherapy alone arm (median PFS 5.1 *vs*. 4.2 months,

P=0.02). Subset analysis showed a greater benefit in patients with squamous histology and thus, a phase III study of ipilimumab in combination with carboplatin and paclitaxel for squamous cell carcinoma of the lung is ongoing (NCT 02279732). Ipilimumab is also being studied in NSCLC in combination with radiation (NCT02239900, NCT02221739) and with other immunotherapy agents (NCT02039674, NCT02174172).

PD-1 inhibitors

PD-1 is expressed on the surface of activated T-cells and has an inhibitory effect on immune response, and multiple therapeutic antibodies against PD-1 are in development. PD-1 inhibitors that have been most extensively studied for lung cancer are nivolumab and pembrolizumab. NSCLC responses to PD-1 inhibition with nivolumab were first reported in a phase I study, with responses seen in 18% of heavily pre-treated NSCLC patients (17). Some of these responses were durable. These results led to two large, phase III trials of nivolumab *vs.* docetaxel in previously treated squamous NSCLC (CheckMate 017, NCT01642004) and non-squamous NSCLC (CheckMate 057, NCT01673867). Results of the CheckMate 017 study were recently reported; patients treated with nivolumab had a statistically significant improvement in OS compared to patients receiving docetaxel (9.2 *vs.* 6.0 months, P<0.001) (10). Nivolumab was FDA approved for previously treated squamous cell carcinoma of the lung based on these data. CheckMate 057 has completed accrual and results are eagerly awaited. A phase III study comparing nivolumab to platinum-doublets for previously untreated NSCLC is currently enrolling patients (NCT02041533).

Pembrolizumab is another PD-1 inhibitor with promising activity in NSCLC. In the recently reported phase I KEYNOTE-001 study, patients with NSCLC were treated with pembrolizumab in different doses and schedules. Most patients (81%) had received prior systemic therapy. Tumor samples from all patients were assessed for programmed cell death ligand of protein 1 (PD-L1) expression prior to treatment. The overall response rate (ORR) in all tumor types was 19.4% with a median duration of response (MDR) of 12.5 months. Median OS was 12 months (11). A phase III trial comparing pembrolizumab to docetaxel for previously treated NSCLC has completed accrual (KEYNOTE-010, NCT01905657); phase III trials in the front-line space are ongoing (KEYNOTE-024, NCT02142738 and KEYNOTE-042, NCT022220894).

PD-L1 inhibitors

An alternate to inhibiting PD-1 is blocking interactions at the ligand level (PD-L1). The two anti-PD-L1 antibodies that are furthest in development for lung cancer are MPDL3280A and MEDI4736. In a phase I study of MPDL3280A, NSCLC patients had a 24% response rate. Some of these responses were long-lasting; a number of patients continued to maintain a response even after the antibody was stopped (12). Larger studies are ongoing: the single arm, phase II BIRCH trial is enrolling patients with PD-L1 positive NSCLC (NCT02031458); the phase III OAK trial randomizes patients with previously treated NSCLC to either MPDL3280A or docetaxel (NCT02008227).

MEDI4736 also has shown promising activity in a phase I study (7), and larger studies are ongoing. The phase III PACIFIC trial is enrolling patients with stage III NSCLC who have completed radiation and randomizing them to either MEDI4736 or placebo (NCT02125461); this is the largest ongoing immunotherapy effort for locally advanced disease. In the phase III ARTIC trial, NSCLC patients who have received multiple prior therapies will be randomized to MEDI4736 *vs.* standard chemotherapy if PD-L1 positive or MEDI4736 in combination with anti-CTLA4 antibody tremelimumab if PD-L1 negative (NCT0235948).

T-cell co-stimulation

"Taking the brakes off" the immune system with checkpoint inhibition has been the major focus of immunotherapy research in NSCLC so far; however, agents that augment co-stimulatory signals are also under study. Ongoing studies are focused on 4-1BB (CD-137), OX40 (CD134), and CD-27 agonists that augment T-cell response, often in combination with checkpoint inhibitors. These three receptors are members of the tumor necrosis factor receptor family and are primarily expressed on T-cells. When activated by interactions with their ligands or with antibodies, these receptors lead to T-cell proliferation and survival, as well as cytokine production (8). Therapeutic antibodies against these receptors are in clinical development.

4-1BB agonists

4-1BB, also known as CD137, is a potent co-stimulatory molecule expressed on activated T-cells, and is not seen

in resting T-cells (13). Interactions between 4-1BB and its ligand lead to T-cell proliferation and activation (18). In pre-clinical models, administration of 4-1BB agonists inhibits tumor growth (19). Urelumab is a fully human anti 4-1BB antibody with agonistic activity. In an initial phase I study, activity was observed in melanoma; no patients with NSCLC enrolled on this trial (20). There is an ongoing phase I/II trial studying the combination of urelumab with nivolumab; this study will enroll patients with NSCLC in an expansion cohort (NCT02253992).

OX40 agonists

Like 4-1BB, OX40 is expressed primarily on activated T-cells and its ligand is expressed on antigen presenting cells (21). When OX40 is activated by either its ligand or therapeutic antibodies, the result is survival and proliferation of T-cells (22). Multiple compounds are in development targeting OX40. MEDI6469 is an anti-OX40 antibody with agonist activity; it is being studied in a phase Ib/II trial as a single agent or in combination with checkpoint inhibition (NCT02205333). An expansion cohort for NSCLC is planned. MEDI6383 is another OX40 agonist currently in phase I studies (NCT02221960).

CD27 agonists

CD27 is another co-stimulatory receptor related to OX40 and 4-1BB. An agonist anti-CD27 antibody, varlilumab, was well tolerated in phase I trials (23). There are ongoing trials enrolling NSCLC patients with varlilumab as a single agent (NCT01460134) and in combination with nivolumab (NCT02335918).

Combination therapy

Striking responses to immunotherapy have been seen; however, with single agent therapy, only a minority of patients will respond. A number of studies are exploring combination therapy in an effort to increase response rates and duration of disease control.

Immunotherapy combinations

As briefly discussed above, ongoing studies are combining multiple immunotherapy agents. Especially promising are combinations of dual checkpoint inhibition with CTLA-4 inhibitors and PD-1 inhibitors. Nivolumab combined with ipilimumab is associated with high response rates in melanoma (24); early phase studies enrolling NSCLC patients are ongoing (NCT01454102). The combination of MEDI4736 and tremilimumab is also under study (NCT02000947). Other studies are ongoing with checkpoint inhibition combined with agents that activate co-stimulatory molecules, such as 4-1BB, CD-27, and OX40.

Immune related toxicity is a concern with these combinations; more than half of all patients treated with a combination of ipilimumab and nivolumab had a grade 3 or 4 treatment-related adverse event (25).

Combinations with chemotherapy

Theoretically, administration of cytotoxic chemotherapy may increase antigen release from tumor cells, potentially increasing efficacy of immunotherapy. A phase II study of ipilimumab combined with carboplatin and paclitaxel suggests improved outcomes (immune-related PFS hazard ratio (HR), 0.72; P=0.05 *vs.* immune-related PFS HR, 0.81; P=0.13) when chemotherapy is administered for several cycles prior to initiation of immunotherapy (9). A number of studies combining checkpoint inhibition with cytotoxic chemotherapy are ongoing (NCT02039674, NCT01454102, and NCT02279732, among others).

Combinations with targeted therapies

EGFR inhibitors have revolutionized the treatment of *EGFR* mutant lung cancer; however, resistance inevitably develops, usually after less than a year (25,26). Studies combining EGFR inhibition with erlotinib or gefitinib with checkpoint inhibitors are ongoing (NCT01454102, NCT0208812).

MEK inhibitors such as trametinib and selumetinib have shown some evidence of activity, especially in *KRAS* mutant NSCLC (27,28). Unfortunately, response rates are low and PFS is short with single agent therapy (28). Trials combining MEK inhibition with immunotherapy in melanoma are ongoing (NCT02224781); trials in NSCLC are likely to follow.

Caution must be exercised when combining targeted agents with immunotherapy. A recent phase I study combining ipilimumab with vemurafenib in metastatic melanoma found the combination to be intolerable, with an unexpectedly high rate of hepatic toxicity (29). This trial shows that unexpected synergistic toxicity may be observed when distinct therapeutics is combined.

Combinations with radiation

Like cytotoxic chemotherapy, radiation can cause tumor antigen release, which may increase efficacy of immune therapy. There have been reports of radiation resulting in response in tumors well outside the radiation field; this is known as the abscopal effect (30), and pre-clinical evidence suggests radiation may increase the efficacy of checkpoint inhibition (31). Ongoing studies are combining checkpoint inhibition with radiation (NCT02239900, NCT02221739, NCT02303990). Toxicity is a concern here as well—pneumonitis is a common and potentially severe toxicity of both immunotherapy and radiation. Patients on these trials will be carefully monitored for pulmonary toxicity.

Biomarker development

Though immunotherapy agents appear to be very active in a subset of NSCLC, many patients will have no response to therapy. Several strategies are being developed to try to select patients most likely to respond to therapy.

PD-L1 staining

Multiple studies have assessed whether expression of PD-L1 can predict for response from checkpoint inhibition. In the phase I study of nivolumab in multiple tumor types including NSCLC, patients whose tumors were positive for PD-L1 (defined as >5% of cells with expression) had a response rate of 36% with nivolumab. None of the patients with PD-L1 negative tumors had a response (17). In the KEYNOTE-001 trial, NSCLC patients with staining for PD-L1 in greater than 50% of cells had longer PFS and OS than those with lower rates of expression when treated with pembrolizumab (11).

The early phase studies with nivolumab and pembrolizumab described above used tumor cell staining for PD-L1 as a marker; Herbst *et al*. examined expression of PD-L1 in both tumor cells and infiltrating lymphocytes in specimens from a phase I study of MPDL3280A (32). Interestingly, they found that expression of PD-L1 on tumor infiltrating immune cells was a significant predictor of response, while PD-L1 expression on tumor cells was not. In patients with NSCLC, the response rate for patients with the highest PD-L1 expression on immune cells (IHC 3) was 83%; the response rate in patients with no staining (IHC 0) was 20%. Of note, a response rate of 20% still compares favorably to response rates seen with cytotoxic chemotherapy for previously treated NSCLC (33).

In summary, studies so far suggest that patients with PD-L1 expression have a higher likelihood of responding to PD-1 or PD-L1 inhibition. These results, however, are all from single-arm trials, and some patients with PD-L1 negative tumors have responded to therapy. The clinical utility of this biomarker has not yet been determined. Ongoing phase III trials randomizing patients to either checkpoint inhibition or standard chemotherapy are incorporating analysis of PD-L1, in addition to other markers. These results should define the role of this marker in selecting patients for therapy. Of note, nivolumab was FDA approved for previously treated squamous cell carcinoma of the lung, without reference to PD-L1 status (10).

Mutational burden

Some of the cancers in which immunotherapy seems particularly active (melanoma, NSCLC, and head and neck squamous cell carcinoma) are cancers with high mutational loads. It has been hypothesized that a high mutation burden correlates with creation of neoantigens, which may be targets for immune cells activated by checkpoint inhibition (34). Several studies have examined the correlation between number of somatic mutations detected by whole exome sequencing and response to immunotherapy agents.

Elegant work by Snyder *et al*. has demonstrated that mutational load in melanoma is associated with clinical benefit to anti-CTLA-4 antibodies tremilimumab and ipilimumab. Specific neoepitopes were identified that predict for benefit—these may resemble epitopes from certain viruses (35).

In the KEYNOTE-001 study in NSCLC, current or former smokers had higher response rates to pembrolizumab than non-smokers (22.5% *vs*. 10.3%) (11). Smokers tend to have far more mutations in their tumors than never smokers; 10 times more in one study (36). Additional studies on tumor samples from this study showed that higher mutation burden was associated with higher rates of durable clinical benefit [partial response (PR) or stable disease lasting longer than 6 months], higher response rates, and longer PFS (34).

Though these results are intriguing, whole genome sequencing has not yet been incorporated into routine clinical care, and mutational burden does not yet have a role in selecting patients for therapy.

Management of immunotherapy related toxicity

The agents described above are generally well tolerated; however, severe toxicities can occur. Pneumonitis, colitis,

and hepatitis have been noted with these agents and can be fatal. Appropriate and timely management of the unique toxicities associated with these agents in critically important; this topic is discussed by Villadolid and Amin in detail in another review in this issue.

Conclusions

It is clear that immunotherapy will play an increasing role in the therapy of lung cancer. Over the next year, the results of several randomized phase III trials comparing checkpoint inhibition with standard chemotherapy will be reported. These studies will help to establish the role of immunotherapy in the treatment of advanced NSCLC. Future research should focus on identification of patients most likely to benefit from therapy, and on rational combination therapy.

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Footnote

Conflicts of Interest: Dr. Carrizosa: Honoraria—Boehringer Ingelheim, Advisory Board—Pfizer. Dr. Gold: Honoriaria—Bristol-Myers Squibb, Advisory Board—Pfizer.

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Combining immunotherapy and radiation therapy for small cell lung cancer and thymic tumors

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Abstract: Recent work with immunotherapy has shown promising results with treatment of several solid malignancies, and there are several reports of good systemic responses with the combination of immunotherapy and radiation therapy (RT), most notably in advanced melanoma. Given the rapid increase in the use of checkpoint blockade as well as anti-tumor vaccines, we review here the preclinical rationale and ongoing clinical work in combining immunotherapy with RT for small cell lung cancer (SCLC) and thymic tumors. While there are several reports of promising results with the combination of immunotherapy and conventional systemic treatment, we focus here on the ongoing clinical studies that combine immunotherapy with RT, and highlight the emerging data for this multimodality approach as well as key preclinical and clinical issues that remain to be addressed. With regards to SCLC, trials exploring to the combination of immunotherapy and RT are already ongoing, but clinical studies for this combination in thymoma are lacking.

Keywords: Immunotherapy; radiation; small cell lung cancer (SCLC); thymoma

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Introduction

Immunotherapy in the context of tumors is a treatment designed to enhance the ability of the immune system to identify and eradicate tumor cells. There has been ample recent evidence to suggest that immunotherapy offers a promising approach for the treatment of several malignancies, notably including solid tumors such as advanced melanoma, renal cell carcinoma, non-small cell lung cancer (NSCLC), as well as hematologic malignancies. Multiple clinical studies are ongoing to examine how best to utilize this promising treatment modality in the context of standards of care (1-4).

The role of the immune system in recognizing and attacking tumor cells has been extensively reviewed in numerous reports elsewhere, most recently by Khalil and colleagues in an excellent overview (5). In brief, it involves the uptake and processing of tumor antigens by antigen-presenting cells (APCs) to activate CD4+ helper T-cells and the ensuing recruitment of CD8+ cytotoxic T-cells to directly kill tumor cells. However, both non-tumor immunomodulatory cells within the tumor microenvironment and tumor cells can directly exert an immunosuppressive role to allow the tumor to evade the immune system. One of these mechanisms is the utilization of checkpoint pathways that otherwise are important to protect normal tissue from inflammatory responses. These result in inhibition of anti-tumor T-cell activation. One of the most promising novel treatment strategies, so-called checkpoint blockade, has been demonstrated to block

this immunosuppression using specific inhibitors against checkpoint receptors such as programmed death 1 (PD-1) on cytotoxic T-cells or programmed death-ligand 1 (PD-L1) on antigen-presenting or tumor cells, so that T-cell activation can uninhibitedly proceed and elicit anti-tumor responses (6). Several other approaches also exist, such as adoptive cell therapy, in which native effector T-cells are harvested, engineered to recognize specific tumor associated antigens, and returned to the patient to elicit the anti-tumor response (7). However, for the purposes of this review, we will primarily focus on checkpoint inhibition and the pre-clinical and clinical data exploring this strategy.

Interest in utilizing radiotherapy and immunotherapy together to exploit synergy of these modalities has grown rapidly (8). Tumor cell death following radiation is known to generate and expose neoantigens, thus modulating the tumor immune microenvironment as well as the systemic immune response (9-12), the topic of several recent reviews (13). Hence, there has been an emergence of trials incorporating RT rationally, combining its immune-potentiating effect with checkpoint blockade to improve anti-tumor immune response locally and systemically, as well as trials utilizing "maintenance" checkpoint blockade after chemoradiation treatment to maintain an immune response. Here, we review the preclinical and emerging clinical rationale for utilizing immunotherapy in SCLC as well as thymic tumors, and review the ongoing clinical studies that utilize immunotherapy in combination with RT, in these two disease areas.

Small-cell lung cancer (SCLC)

SCLC remains one of the most challenging thoracic malignancies. While great advances have been made in NSCLC in identifying molecular targets (i.e., tumors mutant in epidermal growth factor receptor, anaplastic lymphoma kinase) that have extended survival impressively in some patients, such advances in SCLC have remained difficult. SCLC is aggressive, grows rapidly, and early rapid recurrence and metastasis is a hallmark (14). Only about a third of patients present with limited stage disease (LS-SCLC), defined as disease in the hemithorax that can be encompassed by a single conventional RT field; the remainder have extensive stage disease (ES-SCLC) (15,16).

Treatment options have remained unchanged over many years. Etoposide/platinum doublet therapy is the systemic therapy backbone and standard of care for any SCLC, and the pattern of failure is usually characterized by a good upfront response in most patients and early relapse within the first year (17-19). Modifications in the RT approach have been the only contributing factor to improvement in overall survival. These include the timing of RT (concurrent vs. sequential) and the use of twice-daily RT for LS-SCLC, the addition of prophylactic cranial irradiation for both LS- and ES-SCLC, and the addition of consolidative thoracic RT in ES-SCLC (20-23). Despite these improvements survival is dismal, with median overall survival for LS-SCLC of about 25 months, and for ES-SCLC of 10 months; analogous 5-year rates are 10%, and about 2% (24). Hence, even modest improvements with novel approaches have great potential to be superior to the current standard of care.

Immunotherapy rationale

Historically, immunotherapy has been difficult to incorporate in to the treatment of SCLC. There have been a number of negative clinical trials in the relatively recent past, including studies showing that post-treatment maintenance therapy with chemotherapy or biologic agents (alpha and gamma interferons, anti-idiotype BEC2 vaccine) did not improve patient outcomes (25). A dendritic cell-based p53 vaccine showed preliminary evidence of immunogenicity (26) but its company is now obsolete making further development of that vaccine unlikely. An additional study examining 2 anti-idiotype vaccines (11D10 and GD2) was terminated due to lack of drug availability (NCT00045617).

With SCLC specifically, two notable characteristics highlight how checkpoint blockade might be incorporated into treatment. First, SCLC has a high mutational burden, as discovered most comprehensively by whole genome sequencing of a large sample of SCLC patients, nearly all of which were treatment naive (27). Bi-allelic inactivation of TP53 and RB1, sometimes by complex genomic rearrangements, was universal. Furthermore, amongst examined solid tumors, SCLC ranks fourth in the number of somatic mutations, a surrogate for the number of neoantigens that a tumor might present to the host immune system (28,29); the top three in that study were melanoma, NSCLC, and bladder cancer. Promising results with checkpoint blockade with two of those three histologies (notably, melanoma and NSCLC) have been observed, and at least in NSCLC, the mutational load seems to govern sensitivity to PD-1 blockade with improved objective response, durable clinical benefit, and

progression-free survival being associated with higher non-synonymous mutation burden and higher neoantigen burden (30). Hence, given the high mutational load and presumably neoantigen presentation of SCLC, checkpoint blockade may also be an attractive strategy for SCLC.

Second, there are preclinical and clinical data examining T-cell interactions between SCLC and the host immune system. Some in vitro SCLC cell lines appear to secrete IL-15 and induce CD4+ Treg cell mediated immunosuppression (31) and SCLC tumors often contain few infiltrating lymphoid cells (32). Exactly how this is mediated is not clear, but decreased expression of HLA-class I antigen has been reported (33) and the balance between effector and regulatory T-cells distinguishes ES-SCLC from LS-SCLC and predicts recurrence (34). This implies that targeting T-cell suppression may be an attractive strategy, and autologous T-cell infusions in patients with advanced SCLC have shown some preliminary evidence of anti-tumor activity in a small non-randomized cohort study (35).

Additionally, SCLC has long been known to induce paraneoplastic disorders in which the host immune system recognizes and targets antigens present on SCLC tumor cells and also in normal tissue. For example, Lambert Eaton Syndrome results from antibodies directed against shared antigens on SCLC cells and normal neurons, including HuD, HuC, and Hel-N1 (36). It was noted nearly two decades ago in retrospective report that SCLC patients with Lambert-Eaton syndrome had improved OS as compared to those without the syndrome (37). Prospective verification of this is ongoing, but the intermediate report is suggestive of benefit (38).

Despite this suggestive data, a phase II trial in this space examining ipilimumab, an anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA4) monoclonal antibody, in the first-line setting combined with paclitaxel/carboplatin in ES-SCLC showed only a 1-month improvement in immune related progression free survival (accounting for tumor response at index lesions in the face of new lesions), but was otherwise reported as a negative trial (39). The ensuing randomized phase III trial with ipilimumab and platinum/etoposide was negative for OS or PFS gain, and showed no change in objective response rate (ORR) (40).

While disappointing, closer examination might yet provide encouragement. As noted by Riess and colleagues (41), early dropout due to disease progression or toxicity was a major problem. Approximately 15% of randomly assigned patients did not receive the study drug, and only 13% of those assigned to receive ipilimumab lived long enough without progression or toxicity to receive it as maintenance. Likewise, in KEYNOTE 028, just over half of the patients with SCLC screened for PD-L1 expression who were eligible for pembrolizumab, a PD-1 blocker, actually received it (42). Still, this produced a response rate (RR) of 29%, impressive for previously treated ES-SCLC, and comparable to 19–20% RR observed with nivolumab (a humanized anti-PD-1 agent) or 29–30% RR with pembrolizumab in previously treated NSCLC patients (43-45). Hence, overcoming the unique clinical challenges of the ES-SCLC patient population may yet reveal a benefit. As they also state, perhaps priming doses of chemotherapy in ES-SCLC are unable to generate the correct or appropriate level of neoantigen expression to drive functional immunogenicity. Lastly, perhaps a strategy to overcome local microenvironment suppression with anti-PD-1 or anti-PD-L1 antibodies is needed in addition to anti-CTLA-4 targeted therapy in which the priming of cytotoxic T cells is enhanced.

In support of the PD-1/PD-L1 strategy is data from patient samples that noted SCLC cells did not show activation of the pathway, but instead tumor-infiltrating macrophages and tumor-infiltrating lymphocytes (TILs) were activated in ≤50% of the 94 patient samples analyzed, at least suggesting that evaluation of the tumor microenvironment (and not just the tumor itself) should be included in clinical trials (46).

Furthermore, additional evidence comes from the Checkmate 032 study that examined nivolumab with or without ipilimumab in pretreated SCLC patients with progressive disease (47). A RR of 19–23% was observed for the combination of nivolumab and ipilimumab compared to 10% with nivolumab alone, and progression-free survival appears to be improved over nivolumab alone, though this was not the intent of the analysis.

The regimens of single agent and combination therapy with checkpoint inhibitors were tolerable; grade 3 or 4 treatment-related adverse events occurred more often in patients receiving the combination of nivolumab and ipilimumab compared to nivolumab monotherapy. Side effects of nivolumab and pembrolizumab in SCLC were similar to those described in the treatment of other cancers with the possible exception of rare autoimmune events such as limbic encephalitis and myasthenia gravis (MG) (47).

Clinical trials of immunotherapy incorporating radiation in SCLC

While there are several trials of immunotherapy with

Table 1 Ongoing studies incorporating RT and immunotherapy in SCLC

Agent	Phase	Endpoint	Patients (n)	Details	NCT
Ipilimumab + nivolumab	II	Primary: OS; secondary: ORR, PFS, toxicity	LS-SCLC [260]	Open label, randomized to chemoradiation and PCI followed by either observation vs. induction ipilimumab + nivolumab ×4 and maintenance nivolumab	NCT02046733
Pembrolizumab	I	Primary: MTD; secondary: PFS	A: LS-SCLC [9]; B: ES-SCLC [80]	A: open label dose escalation of pembrolizumab with concurrent chemoradiation (platinum/etoposide ×4, 150 cGy BID to 45 Gy) followed by maintenance pembrolizumab; B: platinum/etoposide chemotherapy for up to 6 cycles followed by consolidative thoracic RT 300 cGY daily to 45 Gy, with concurrent pembrolizumab for cycle 3 and onward followed by maintenance pembrolizumab	NCT02402920
Tremelimumab + durvalumab	II	Primary: PFS; secondary: irRR, OS	Recurrent SCLC [20]	Open label, randomized to tremelimumab + durvalumab with or without SBRT immediately preceding immunotherapy	NCT02701400

SCLC, small cell lung cancer; OS, overall survival; ORR, objective response rate; PFS, progression-free survival; MTD, maximum tolerated dose; LS-SCLC, limited stage disease; ES-SCLC, extensive stage disease; PCI, prophylactic cranial irradiation; BID, twice daily; SBRT, stereotactic body radiation therapy; NCT, National Clinical Trials number.

systemic therapy in SCLC that have been reviewed just recently (48), here we will focus on the three ongoing studies that include RT (*Table 1*).

The first focusing on LS-SCLC is the phase II STIMULI trial, incorporating induction with concurrent ipilimumab and nivolumab at about 6–8 weeks following the fourth cycle of standard of care concurrent chemoradiation for LS-SCLC (49). All patients will also undergo prophylactic cranial irradiation following chemoradiation. The induction phase will incorporate four infusions of both checkpoint blockers, to be then followed by maintenance nivolumab alone. The strategy is based on the rationale of combining ipilimumab's peripheral T-cell priming to increase intratumoral T-cells with maintenance PD-1 blockade to then attempt to sustain this activated cell population. One major question will be whether four cycles of chemotherapy with concurrent radiation will lead to suboptimal neoantigen presentation and priming by the time ipilimumab is delivered 2 months following the last dose of therapy, which might not yield the benefit of administrating ipilimumab earlier in the regimen. This study will be open across multiple centers in Europe and is estimated be completed in 2019.

Similarly, a single-institution phase I dose-escalation study is underway at M.D. Anderson for pembrolizumab given with concurrent RT in two populations (50). The first will include patients with untreated LS-SCLC, and notably, pembrolizumab will be administered upfront with concurrent chemoradiotherapy with platinum/etoposide for standard four cycles, with twice daily RT to the chest in 150 cGy fractions for a total dose of 45 Gy. The second group will include patients with untreated ES-SCLC for whom the treatment paradigm will include pembrolizumab starting with cycle 3 of chemotherapy. RT will be the same dose as the LS-SCLC group, but the timing of this is left open at this point, likely given that the ES-SCLC population will be very heterogeneous and the timing of RT will depend on the response to chemotherapy as well as the functional status of each patient. Given that this is a phase I trial, dose limiting toxicities will be the main focus to judge whether concurrent RT in this potentially ill population will be feasible, with helpful data on pulmonary function and toxicities exacerbated by radiation, but this should provide data for the design of future studies to move checkpoint blockade agents in the first-line setting, and whether twice daily RT in combination with checkpoint inhibition is a manageable strategy.

Both of the above studies will be primarily useful to help judge the effectiveness of the immunotherapy, and perhaps some measures on whether RT augments this in the ES-SCLC population. However, a recently-opened randomized phase II study at Emory University explores whether RT has any impact in eliciting an abscopal effect in SCLC, defined as response in lesions away from that

which received local therapy. It combines dual checkpoint inhibition using tremelimumab and durvalumab, anti-CTLA4 and PD-L1 antibodies respectively, with or without RT (51). In the arm without RT, infusion is every 4 weeks, but in the RT arm, this is preceded by stereotactic body radiation therapy (SBRT) or hypofractionated RT, either daily for 5 days or for three fractions every other day. The primary outcome measure is PFS, and secondary endpoints include the immune-related RR. Crucially, the protocol also specifies paired biopsies at baseline, end of cycle 2, and at progression to characterize evolution in TILs and PD-1/PD-L1 expression, and will allow study for whether this combination approach in SCLC patients shows similar synergistic efficacy as has been seen in melanoma (52).

These are the three main ongoing studies that focus on intersecting RT with immunotherapy in SCLC, and will help to inform basic questions on tolerability, response, and elucidate the intratumoral and circulating host immune responses to immunotherapy and RT combinations. Additional questions on the optimal dose of immunotherapy, dose and fractionation of RT, selection of radiation targets, and how to time the two treatment modalities will remain for future studies. The approach in nearly all cases is empiric, however, there is no solid data at this time to suggest the superiority of one of these combinations over another in any disease, including SCLC.

Thymic tumors

Thymic tumors represent a heterogeneous group of rare conditions, accounting for less than one percent of all malignancies. Ninety percent of all thymic tumors are thymomas. The 5-year survival rates for thymomas are well above 70% (53,54). However, locally-advanced thymomas frequently recur in a more disseminated distribution, mostly in the pleural space, a challenge that is frequently addressed with local therapies such as surgical resection or RT, yet not always with long-term success. For example, in a series of 156 patients with stage II–IV thymoma treated with definitive or adjuvant RT across two large institutions, failures out of the radiation field were the most common, and the five-year failure rate was 24% (55). Still, postoperative RT in stage II and III thymoma does appear to portend an OS benefit in a large multi-institutional retrospective analysis of 1,263 patients (56).

Thymic carcinomas constitute the most common other histology of thymic tumors, a different disease that is much more aggressive and often treated with tri-modality therapy (57-59). Patients treated with such an aggressive approach appear to have better survival compared with less aggressive treatment among patients with stage III disease (60). In a large international analysis of 1,042 patients with thymic carcinoma, a margin-negative resection and adjuvant RT were important for OS. Still, recurrence rate at 5 years was about 35% and median OS was 6.6 years (61). Systemic therapy is typically with platinum-based treatment, offering significant disease response and palliation of symptoms in advanced patients (62).

The rare nature of thymic tumors has made large-scale preclinical data difficult to generate. This led to the creation of the International Thymic Malignancy Interest Group (ITMIG) and the development of a large, centralized database across 50 institutions with over 6,000 cases at present (63). This collaboration has allowed for consensus guidelines to be developed for classification of thymoma (64), a proposal for standardized TNM staging of thymic tumors (65,66), and response criteria to judge the effectiveness of RT (67).

With regards to immune interactions, thymomas have long been known to interact with T-cell development. For example, an association with MG, a disorder characterized by chronic muscle weakness and antibodies targeting a particular peripheral postsynaptic nicotinic acetylcholine receptor, has been long known. Nearly two decades ago, in a series of 20 patients with thymoma with MG, a higher prevalence of a particular CTLA-4 polymorphic allele with a 3'-untranslated region (AT)n-repeat polymorphism was noted as compared to non-thymoma related MG (68). However, in that study, there was no group of thymoma patients without MG. MG in thymoma seems to be dependent on export of autoreactive CD4+ T-cells, though the exact mechanism still seems to be unclear (69). Associations between CTLA-4 alleles and MG in thymoma patients have been found in populations of Caucasian Germans, as well as Chinese patients (70,71). Still, how this actually impacts the pathogenesis of thymoma, MG, or thymoma-associated MG is not known. Lastly, how CTLA-4 expression impacts patient outcome remains unknown.

Furthermore, the molecular biology of thymic tumors is only just beginning to be understood. Very few cell lines exist; three are from thymic carcinoma patients, and the first reports of B1 and AB lines are still relatively recent (72). While genomic aberrations have been known for some time (73,74) in that specifically B3 thymomas display extensive chromosomal imbalances, only one study has performed

whole genome sequencing (75) and did not identify any mutations of well-characterized cancer genes. Hence, little molecular biology is known about how to integrate immunotherapy approaches for thymomas, for example in the mutational load of thymoma patients.

A few studies have examined the PD-1/PD-L1 pathway in thymic tumors. One Japanese study investigated PD-L1 expression measured by immunohistochemistry and found that PD-L1 expression was significantly higher in thymic carcinomas than in thymomas (76). Data from Stanford University on 69 tumors and 17 normal controls showed strong staining of the epithelial component of thymic tumors, with a higher prevalence in B1-3 thymomas and thymic carcinomas; there was some suggestion of worse prognosis in the PD-L1 positive patients (77). Another study from Japan similarly suggested that PD-L1 expression was associated with more advanced Masaoka stage at presentation and WHO type B2 or B3 thymoma, as well as worse disease-free survival (78). The same group also found that 3 of 11 patients with thymic carcinoma showed increase in PD-L1 copy number, and increased expression in these patients correlated with improved OS (79). Lastly, PD-L1 expression was also found in a French series of 104 patients, again confirming the previous data (80).

Another report examining tissue from 15 patients with thymic carcinoma noted strong PD-L1 immunostaining in a third of patient samples (81). Lastly, one anecdotal report also exists showing complete radiographic response to anti-PD-1 therapy in a patient with metastatic thymic squamous cell carcinoma (82), as well as one that noted an abscopal response in a patient with thymic carcinoma who was pre-treated with granulocyte-macrophage colony-stimulating factor and underwent RT to a single metastasis (83).

Outside of checkpoint blockade studies, alternative immune-directed approaches using specific antigenic targets only recently have begun to show promise. The largest series of thymic epithelial tumors to be examined for expression of mesothelin, a surface antigen present on normal mesothelial cells lining the pleura, peritoneum and pericardium, noted a majority of thymic carcinomas (79%) and only a small fraction of thymomas (10%) showed expression (84). In thymic carcinoma, a third showed nearly uniform tumor cell expression, and notably, those with high expression had improved OS. Specific mesothelin-targeted treatment has shown promise in both mesothelioma (with an anti-mesothelin immunotoxin) as well as advanced pancreatic and ovarian cancer (antibody-drug conjugate), suggesting this may be a promising target in thymic carcinoma as well (85,86).

Clinical trials of immunotherapy in thymic tumors

Currently there are no clinical trials that combine RT with immunotherapy for thymic tumors. Indeed even those examining immunotherapy alone are few. To our knowledge, only two trials have reported early results with immunotherapy in thymic tumors.

Early results from a phase II study on pembrolizumab in patients with recurrent thymic carcinoma reported that 30 patients showed an objective RR (ORR) of 24%, with five patients with partial response and an additional 10 with stable disease; nearly all of those patients that responded are now beyond 15 cycles of drug (NCT02364076) (87). Of note, an unusual spectrum of autoimmune disorders accompanying this therapy was noted, including polymyositis/myocarditis and type 1 diabetes, manifesting as grade 3 asthenia, complete heart block, and grade 4 hyperglycemia. All recovered with discontinuation of drug and with steroids. Additional studies with pembrolizumab in thymoma are open at MDACC (NCT02721732) and in Republic of Korea (NCT02607631).

Another early report on a phase 1 study of avelumab, a fully human IgG1 anti-PD-L1 antibody, in advanced thymoma (NCT01772004) (88) found that in a cohort of 7 thymoma and 1 thymic carcinoma patients, 4 patients had a partial response (all thymoma), and the only thymic carcinoma patient had stable disease. Similarly as in the study on pembrolizumab, uncommon immune-related adverse events were observed in five patients, including asthenia, myalgia and myositis, among others, and three of the four responding patients had myositis.

Still, immunotherapy in thymoma is only in its infancy. The early preclinical and clinical data appear to be promising, but our understanding of how thymic tumors interface with the host immune system remains poor, largely due to the difficulty of studying the disease owing to its clinical rarity and the availability of few in vitro models. Aside from data on response, these pioneering studies will provide crucial correlative and exploratory data that will allow further investigation of the basic biology of thymic tumors and their interaction with the immune system that will finally allow us to understand this disease better and ultimately provide better specialized and more effective treatment.

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Footnote

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Integrating immunotherapy into chemoradiation regimens for medically inoperable locally advanced non-small cell lung cancer

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Abstract: For patients with inoperable stage II–III non-small cell lung cancer (NSCLC), the backbone of curative intent therapy is concurrent chemoradiotherapy (CRT). As checkpoint inhibitors have shown clinical benefit in the setting of metastatic NSCLC, additional study is necessary to understand their role in patients receiving CRT. When integrating immunotherapy with radiotherapy (RT) for cure, clinicians will need to consider synergy, timing, doses, and safety among the combination of therapies. This article seeks to review data evaluating interactions, temporal sequencing, fractionation, and overlapping toxicity profiles of thoracic chemoradiation and immunotherapy.

Keywords: Immunotherapy; chemoradiation; non-small cell lung cancer (NSCLC); PD-1; PD-L1

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Introduction

For patients with inoperable stage II–III non-small cell lung cancer (NSCLC), the backbone of curative intent therapy is concurrent chemoradiotherapy (CRT). The supportive evidence for the use of combined CRT is rooted in several decades of methodical clinical studies that established the superiority of concurrent CRT compared to either modality alone or sequential delivery of chemotherapy followed by radiotherapy (RT) (1-5). The customary platform for localized and inoperable NSCLC consists of concurrent chemoradiation with a platinum-based doublet and 60 Gy of RT delivered daily over 6 weeks followed by consideration of two cycles of consolidative chemotherapy, particularly for carboplatin and paclitaxel regimens (6,7). Although consolidative chemotherapy was not found to demonstrate an obvious survival benefit for inoperable, locally advanced NSCLC (8,9), its incorporation into RTOG 0617 has led to its acceptance as the de facto standard of care (7). Despite its acceptance as a curative intent treatment, concurrent CRT results in relatively meager treatment outcomes with median survival rates of 20–28 months and 5-year overall survival (OS) rates of 15–20%.

The advent of novel immunotherapy agents affords patients and clinicians therapeutic modalities to improve patient longevity and avenues to study innovative combinations of therapies (10-13). Incorporation of immunotherapy with standard therapy provides the potential to build upon the gains of the well-established regimen of CRT for inoperable NSCLC. Since checkpoint inhibitors have shown clinical benefit in the setting of metastatic NSCLC, additional study will be necessary to understand their role in combined modality CRT. When integrating immunotherapy with RT for cure, clinicians will need to consider synergy, timing, doses, and safety among the combined therapies. This article seeks to review data evaluating interactions, temporal sequencing, fractionation, and overlapping toxicity profiles of CRT and

immunotherapy.

Immune modulation in the setting of RT

Local ionizing radiation can interact with the host's immune system by increasing the tumor antigen specific effector cells that traffic to a tumor. In a study comparing xenografts with B16-F0 tumors, irradiated mice (treated with 15 Gy) had greater ability to present tumor antigens and specific T-cells and tumor infiltrating lymphocytes than non-irradiated mice (14). In melanoma murine models, tumor control increased with the size of the RT dose as did tumor-reactive T cells, but a dose of 7.5 Gy per fraction proved to be the regimen with the optimal tumor control and tumor immunity with the lowest number of T-regulatory cells (T-regs) (15).

RT modulates the immune system and can help to mount an immune response that can result in immunogenic cell death. Radiation releases tumor antigens and facilitates tumor antigen release by dendritic cells (DC) and cross-complementation on major histocompatibility complex-1 (MHC-1) (16). RT potentiates calretinin's exposure on the cell surface and release of ATP and high mobility group box 1 (HMGB1), which seems to be required for DC activation and immune priming against malignant cells (17). Therefore, RT also acts as an in-situ tumor vaccine and may immunize the patient against their neoplasm and can provide immunologic memory which may endure for the host's lifetime (18,19).

RT also provides a pro-immunogenic effect on the tumor microenvironment (18). RT elicits activation of both innate and adaptive immunity (20), and these immune responses are potentiated by the cellular damage caused by RT and the cascade of interleukin-1β (IL-1β), tumor necrosis-factor-α (TNF-α), and chemokine (C-C-C motif) ligand 16 (CXCL16), MHC molecules, adhesion molecules and death receptors (21). RT also can reprogram macrophage differentiation to an iNOS+/M1 phenotype that orchestrates effective T cell immunotherapy (18,22).

The interaction of RT and checkpoint inhibitors or other immunotherapies may lead to an abscopal effect whereby after the administration of RT to one location, a non-responding systemic tumor then displays diffuse systemic response at distant sites from the site of irradiation. This concept has gained much attention in the setting of metastatic melanoma patient who had been maintained on ipilimumab with relatively stable disease subsequently received stereotactic body RT to an enlarging paraspinal mass and was found to have response at their other sites of distant metastases. The authors suggested that the tumor was resistant to T cell mediated antitumor effects until the delivery of RT (23). T cells are thought to be a driver for the abscopal effect, which may require Flt-3 ligand as mice bearing tumor in both flanks responded in both flanks despite irradiation of only one flank when Flt-3 was available (24). This abscopal effect has been demonstrated on multiple occasions but is thought to be relatively infrequent, and no reliable method has been discovered to reproducibly harness these potent series of events clinically.

PD-1 and its interaction with RT

Programmed cell death-1 is an immune checkpoint inhibitory receptor and facilitates immune escape (25). PD-1 primarily curbs the activity of T cells in the periphery during chronic inflammation, infection or cancer and limits autoimmunity. When PD-1 interacts with its ligand PD-L1, it can inhibit T cell growth, survival, and effector function, such as cytokine release and cytotoxicity (26), and leads to tumor specific T cell apoptosis (27), stimulates the differentiation of CD4+ T cells into T-regs (28) and allows for the resistance of tumor cells to cytotoxic T cell (CTL) attack (29).

Inhibition of PD-1, and likely PD-L1, improves tumor rejection. Polyclonal antibody against PD-L1 can promote tumor rejection in models (29). Since PD-1 is expressed directly on tumor surfaces, this is an attractive target for immune-mediated responses. PD-1 blockade can allow for tumor rejection and immune-mediated signaling to allow the immune system to attack the tumor. PD-1 expression is generally increased in tumors with a higher non-synonymous mutational burden in tumors and is associated with improved responses and durable clinical benefit with longer progression-free survival in NSCLC (30). Given the high mutational burden that is often seen in smokers who develop NSCLC, PD-1/PD-L1 inhibition appears to be a logical combination.

Blockade of PD-L1 improves T cell responses leading to tumor rejection (31). PD-L1 can be upregulated in the tumor microenvironment after RT in murine models. The addition of anti-PD-L1 therapy can improve the efficacy of RT through a CTL-dependent mechanism. This combination also reduced tumor-infiltrating myeloid-derived suppressor cells that contribute to altering the tumor microenvironment (32).

Importantly, the interaction of stereotactic RT can

Table 1 Trials of PD-1 or PD-L1 agents combined with chemoradiation for NSCLC

Clinical trial title (phase of trial)	NCT identification (number/institution/status)
Pembrolizumab, Paclitaxel, Carboplatin, and Radiation Therapy in Treating Patients With Stage II-IIIB Non-Small Cell Lung Cancer (I)	NCT02621398/Rutgers Cancer Institute of New Jersey, University of Pennsylvania, Yale/recruiting
Consolidation Pembrolizumab Following Chemoradiation in Patients With Inoperable/ Unresectable Stage III NSCLC (II)	NCT02343952/Hoosier Oncology Group/active, not recruiting
A Global Study to Assess the Effects of MEDI4736 Following Concurrent Chemoradiation in Patients With Stage III Unresectable Non-Small Cell Lung Cancer (PACIFIC) (III)	NCT02125461/AstraZeneca/active, not recruiting
Nivolumab COnsolidation with Standard First-line Chemotherapy and Radiotherapy in Locally Advanced Stage IIIA/B Non-Small Cell Lung Carcinoma (NICOLAS) (II)	NCT02434081/European Thoracic Oncology Platform/recruiting
Cisplatin and Etoposide Plus Radiation Followed by Nivolumab/Placebo for Locally Advanced NSCLC (III)	NCT02768558/RTOG Foundation, Inc./recruiting
MPDL3280A with Chemoradiation for Lung Cancer (II)	NCT02525757/MD Anderson Cancer Center/ recruiting

Ongoing trials incorporating immunotherapy with definitive chemoradiation for inoperable NSCLC.

augment antigen-specific PD-1 mediated antitumor responses by inducing a more robust immune response and cross-presentation of tumor antigen, which was studied in melanoma and breast cancer models (33). In those models, RT resulted in the development of antigen-specific T cell and B cell-mediated immune responses. These immune stimulating effects of RT were increased when RT was combined with anti-PD-1 therapy or regulatory T cell depletion and resulted in improved local control of the tumor.

As discussed in other articles in this series, anti-PD-1 and anti-PD-L1 therapies have shown clinical activity for NSCLC alone and in combination with chemotherapy. Since the clinical effect of anti-PD-1/anti-PD-L1 therapies is evident without the incorporation of RT, it is plausible that the incorporation of RT may provide combinatorial, abscopal or synergistic effects.

Timing, dose, fractionation of immunotherapy with chemoradiation

To date, clinicians have related many of the abscopal responses to hypofractionated irradiation regimens, often with stereotactic body radiotherapy (SBRT), also termed stereotactic ablative radiotherapy (SABR). Dewan et al. evaluated three RT fractionation schemes: 20 Gy × 1, 8 Gy × 3 or 6 Gy × 5 with or without CTLA blockade. CTLA blockade alone was ineffective, but when combined with any of the RT regimens, growth delay was seen. Abscopal effects were evident with the combination of the fractionated RT designs (34). Clinically, abscopal effects have been seen with 8 Gy × 3, 6 Gy × 5, and 9.5 Gy × 3 fractions (23,35,36). The greatest difference occurred for patients with 8 Gy × 3, and 80% of tumors outside the field regressed (16). Lower RT doses may cause reprogramming of macrophages toward an iNOS+/M1 phenotype, enabling them the ability to allow tumor rejection (37).

Also, concurrent platinum and RT cause calretinin translocation from dying tumor cells at dosages tested in a dose-dependent manner. Calretinin translocation increased due to platinum but remained stable after adding RT. Nevertheless, platinum and RT cause release of HMGB1 from dying tumor cells. When RT was combined with paclitaxel, adding RT caused immunogenic cell death (18).

A study by Gulley *et al.*, demonstrated the possible efficacy of standard radiation fractionation of 1.8–2 Gy per day in combination with a poxviral vaccine. T cell responses were seen in the tumor antigens and not in the vaccine, suggesting irradiation promoted the activation of T cells (16,38). Therefore, the combination of standard fractionation CRT and immunotherapy may be effective when they are used together.

Ongoing trials will help to elucidate the role and timing of PD-L1 or PD-1 blockade for inoperable NSCLC treated with definitive chemoradiation (*Table 1*).

Additional immunotherapy combinations

For locally advanced NSCLC, other agents have been

investigated, including tecemotide (L-BLP25), a mucin 1 (MUC1) specific agent that induced T cell responses to MUC1. The phase III START trial was a double-blind phase III trial that randomly assigned 1,006 subjects to tecemotide and 507 to placebo. Median overall survival (OS) was 25.6 months with tecemotide *vs.* 22.3 months with placebo (HR 0.88, 95% CI: 0.75–1.03, P=0.123). In patients who received prior concurrent CRT, median OS for those who received tecemotide was 30.8 months compared to 20.6 months for the control group (HR 0.78, P=0.016), whereas patients who received sequential CRT did not benefit in terms of OS (39,40). In the group of patients who received prior CRT, high soluble MUC1 and antinuclear antibodies correlated with tecemotide benefit (41). However, in a subsequent study by Katakami *et al.*, which randomized Japanese patients (n=172) with stable or clinical responses after CRT to receive adjuvant tecemotide vs. placebo, no apparent trend toward increased OS or other secondary endopoint with tecemotide was observed (42).

Additionally, study of GV1001, a telomerase peptide vaccine, was administered after CRT in a phase I/II trial of 23 patients. A GV1001-specific immune response developed in 16/20 evaluable patients and long-term immunomonitoring showed persisting responses in 13 patients. Immune responders demonstrated a median progression-free survival of 19 months compared to 3.5 months for nonresponders (P<0.001). Responders all harbored durable GV1001-specific T-cell memory responses with high IFNγ, and low IL-4 and IL-10 levels (43,44).

Toxicities of immunotherapy overlap with RT side effects

Administration of thoracic RT places patients at higher risk of radiation-induced pneumonitis, and the clinical presentation is similar to immunotherapy-induced pneumonitis with dry cough, fever, dyspnea, and tachycardia. Urgent initiation of steroid therapy is often required. A study evaluating 915 patients who were treated with PD-1/PD-L1 antibody demonstrated that 43 patients developed pneumonitis (about 5% of patients). Pneumonitis was more likely to occur when anti-PD-1/anti-PD-L1 and another simultaneous immunotherapy were administered, such as concurrent CTLA-4 therapies. Pneumonitis is a toxicity of variable onset clinically, and in the aforementioned study, it ranged from 9 days to 19.2 months (45). Concern for a pneumonitis requires urgent evaluation with imaging and often rapid initiation of steroids to avoid severe and potentially life-threatening respiratory compromise.

Another complication that can occur with both RT and immunotherapy is myocarditis. Unlike pericarditis that can occur in the acute or subacute setting, radiotherapeutic injury to the myocardium is thought to be a delayed effect with long-term toxicities such as coronary artery disease and valvular injury. In contrast, fulminant cases of myocarditis from immunotherapy have been described, particularly with the combination of nivolumab and ipilimumab. The incidence of fatal myocarditis with nivolumab alone is <0.01% and with dual nivolumab and ipilimumab is 0.17%. The incidence of any myocarditis with single-agent nivolumab is 0.06% compared to 0.27% with dual agent therapy. In post-mortem examination of the cardiac tissue of immune checkpoint mediated myocarditis, increased expression of PD-L1 was found in the injured myocardium of patients, consistent with the upregulation of myocardial PD-L1 studies in mice. Investigators have hypothesized that PD-L1 upregulation in the myocardium is a cytokine-induced cardioprotective mechanism that is abrogated by immune checkpoint blockade (46).

Therefore, combinatorial therapy of CRT and immunotherapy must be approached with caution and careful clinical evaluation in prospective clinical trials.

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Footnote

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First-line nivolumab (anti-PD-1) monotherapy in advanced NSCLC: the story of immune checkpoint inhibitors and "the sorcerers apprentice"

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Non-small cell lung cancer (NSCLC) has usually been thought to be a non-immunogenic tumor, because early studies with Bacillus Calmette-Guerin, interleukin-2, or interferon have failed to demonstrate any benefit in NSCLC. Recently, some studies indicated that immune mechanisms play a vital role in the origin and development of lung cancer, and the abnormality of immune checkpoints would be the chief culprit. Immune checkpoint-inhibitors have shown promising activity in several solid tumors, including NSCLC. But interfering with the complex immune system in tumor immunosurveillance can trigger not only long lasting responses, but also severe and sometimes irreversible immunological side effects, as seen with the first approved cytotoxic T-lymphocyte antigen-4 (CTLA-4) inhibitor ipilimumab. This reminds one of a famous German poem written by Johann Wolfgang von Goethe in the year 1797, where an apprentice takes the opportunity to "play a little" with some magic when his master wizard had gone out of the house. But the apprentice summoned some powerful spirits he couldn't actually control.

Better understanding of the immune system and identification of potential new targets in the immune checkpoint pathway has led to development of new compounds targeting programmed cell death-1 (PD-1), programmed cell death-ligand 1 (PD-L1) or other immune checkpoint molecules with different efficacy and toxicity profiles.

In a phase I study of patients with solid tumors, nivolumab (1)—a fully human IgG4 monoclonal antibody—was given biweekly in escalated doses for up to 12 cycles over 2 years. In the NSCLC cohort of 129 heavily pretreated patients (55% receiving at least 3 prior lines of therapy) the overall response rate (ORR) was 17% with a median duration of response of 74 weeks (range, 6.1-133.9 weeks). The median survival was 9.9 months with 1- and 2-year survival rates of 42% and 24%, respectively. However, the median progression free survival (PFS) was only 2.3 months. Side effects included skin (20%), gastrointestinal (15%) and pulmonary (9%) toxicities, being the most commonly observed adverse events (AEs). A lower frequency of gastrointestinal toxicities (2%) was seen (grade 3/4) compared to ipilimumab (20%). However, pneumonitis was reported in 6% (8/129) of patients with two deaths (2). PD-L1 expression analysis was performed in 49% (63/129) of patients and was defined as expression in at least 5% of tumor cells on immunohistochemistry (IHC). PD-L1 positivity was found in 49% (31/63) of patients. The ORR in patients with PD-L1 positive and PD-L1 negative tumors was 16% and 13%, respectively, showing that PD-L1 status was not a good predictive marker in this study.

At the ASCO annual meeting 2014 new data have been presented for nivolumab monotherapy as 1st-line treatment compared to standard chemotherapy (3). In this study patients with squamous or non-squamous advanced NSCLC received nivolumab 3 mg/kg IV Q2W until progression or unacceptable toxicity. Primary endpoint was safety; secondary endpoints included ORR and PFS rate at 24 weeks. Interim analysis of the first 20 patients was presented showing a tolerable safety profile. It is notable that no pneumonitis occurred among those 20 patients. 20% experienced grade 3/4 toxicities, being mainly elevated liver enzymes. However,

only two patients discontinued treatment due to treatment related AEs. ORR was 30% with the median of duration not being reached. In this study no response was seen in those patients defined as PD-L1 negative. However, only 7 PD-L1 negative patients were treated so far. The effect of PD-L1 status might be different in those two studies, the reason for that remain unclear. The methods used to measure the expression of PD-L1 may be responsible. Also the baseline characteristics of included patients between the two studies, such as prior therapy or tumor stage, were different.

In conclusion, the experience with the PD-1-inhibitor nivolumab is still preliminary. Response rates are promising even in heavily pretreated patients and are in the range of other PD-1 and PD-L1 antibodies. It is noteworthy, that duration of responses is uncommonly long with these agents. However, the toxicity profile still remains to be defined in larger patient cohorts. Especially pneumonitis, which is a common side effect in many drugs (e.g., gefitinib, erlotinib, gemcitabine), needs to be monitored carefully, particularly when nivolumab is combined with other agents.

In general, many questions related to immunotherapy remain unanswered: is inhibition of PD-1 or PD-L1 the better approach? What is the ideal schedule and duration of therapy, and should we combine immunotherapy with targeted therapies or chemotherapy? Is PD-L1 status a good predictor of efficacy? To answer these questions we have to wait for the results of several ongoing studies. Until that we may sometimes find ourselves in the position of the sorcerers apprentice from the above mentioned poem, when at the end he shouted for the big sorcerers help: "I have need of Thee! From the spirits that I called Sir, deliver me!"

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Footnote

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Preclinical rationale for combining radiation therapy and immunotherapy beyond checkpoint inhibitors (i.e., CART)

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Abstract: An increasing appreciation for the role of the immune system in targeting cancer cells over the last decade has led to the development of several immunomodulatory agents aimed at enhancing the systemic antitumor immune response. One such method is the use of T cells that are genetically engineered to express chimeric antigen receptors (CARs). The remarkable success of this approach in advanced hematologic malignancies has garnered much enthusiasm for using this novel tool in treating other cancers. However, multiple challenges have hampered the application of this therapy to a broader set of solid tumors, most notably lung cancer. Immunotherapy has already shown great success in lung cancer, and is now the first-line treatment in PD-L1 expressing metastatic disease. Given the mounting evidence that radiation therapy plays a crucial role in amplifying the immune response elicited by immunomodulatory agents, there is potential for radiation to help in overcoming some of these challenges. In this review, we describe the basic principles of CAR T cell therapy and examine its successes and challenges to date. We then discuss the preclinical and clinical data supporting the use of radiation with immunomodulatory agents with a focus on preclinical rationale for combining CAR T cells and radiation therapy in future experiments with a focus on lung cancer.

Keywords: Chimeric antigen receptor T cells (CAR T cells); immunotherapy; radiation therapy

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Introduction

In the United States, lung cancer is the second most frequently diagnosed cancer, affecting almost 225,000 people annually. It is one of the most lethal cancers resulting in over 150,000 deaths annually (1). While incremental progress has been made with combined modality treatment including surgery, radiation, and chemotherapy over the last few decades, lung cancer remains difficult to treat in its localized and distant forms with five years survival rates of 54% and 4%, respectively (2). In recent years, the introduction of several agents targeting specific oncogenic signaling pathways such as EGFR, or ALK provide additional tools to manage non-small cell lung cancer and prolong survival in a subset of patients harboring specific genomic alterations.

More recently, increasing appreciation for the role of immune surveillance in controlling aberrant cell growth has led to the development of several immunotherapy agents in various cancers including lung cancer. These agents inhibit the ability of cancer cells to evade the immune system by blocking immune checkpoint receptors such as CTLA-4 or PD-1 that normally down-regulate antitumor T cell activity upon binding to their respective ligands. Recent results in clinical trials have shown dramatic responses; for

example, the phase III CheckMate 057 study of an anti-PD-1 monoclonal antibody, nivolumab, demonstrated a median survival of 12.2 months compared to 9.4 months with docetaxel in patients with advanced non-squamous non-small cell lung cancer previously treated with platinum-based doublet therapy (3). CheckMate 017 revealed a 9.2-month median overall survival in patients with squamous non-small cell lung cancer treated with nivolumab compared to 6.0 months with docetaxel when administered as second line therapy (4).

Despite the marked benefit, however, the efficacy of such agents is limited to a specific subset of patients. In CheckMate 057, while the overall objective response rate was only 19%, the response rate in patients with >1% PD-L1 expression was much higher at 38%. The median survival for this subset of PD-L1 expressing patients was also significantly higher at 17.7 months compared to 9.0 months in patients with <1% PD-L1 expression (3). The results suggest that the efficacy of these agents is limited, at least in part, due to significant heterogeneity in the expression of specific checkpoint ligands. In contrast, in Checkmate 017, there was no correlation between PD-L1 expression and response or survival even though 47% of tumor specimens in the nivolumab arm expressed PD-L1 ≥1%, (4). Furthermore, a pooled analysis of non-small cell lung cancer patients treated with nivolumab revealed that while PD-L1 expression is correlated with a greater response, patients with PD-L1 expression <1% also demonstrated improved survival when treated with nivolumab as compared to docetaxel (5). Therefore, while response and survival can correlate with PD-L1 expression, alternative markers of response are necessary to identify the subset of patient that are likely to benefit. Moreover, given that only a minority of patients benefit from nivolumab, alternative immunotherapy options are necessary in this disease, or consideration of combination immunotherapy with multiple agents and/or with radiotherapy.

A promising approach for optimizing the anti-tumor immune response that has garnered significant enthusiasm over the past few years utilizes genetically engineered T lymphocytes with tumor-specific chimeric antigen receptors (CARs). While it has been more than 25 years since Gross *et al*. first proposed the concept of using CAR T cell therapy to combat tumors, recent advances in receptor design, synthesis, and T cell expansion and delivery mechanisms have produced remarkable results in patients with hematological malignancies (6-12).

In this paper, we will briefly review the preclinical advances in CAR T cell therapy and clinical results in hematological malignancies, where there is the greatest available data. We will then discuss the major challenges that limit the application of CAR T cell therapy in the clinic, particularly for solid tumors. Finally, we will discuss the rationale for combining CAR T cells with radiation therapy and the potential for treatment synergy that may help overcome these challenges.

Chimeric antigen receptor modified T cells (CAR T cells)

Evading immune surveillance is one of the hallmarks of cancer (13). A major mechanism by which cancer cells trick the tumor antigen handling apparatus of the adaptive immune system is by down-regulating the expression of class I major histocompatibility complex (MHC) proteins (14). Without proper recognition of immunogenic epitopes presented on MHC molecules, cancer cells can escape cytotoxic T cell mediated responses. One approach to counteract this is by engineering T cells to express CARs that are able to recognize tumor antigens with high specificity in an MHC-independent manner. In this approach, T cells are first harvested from a patient by apheresis, purified, and then genetically engineered to express CARs specific for a cancer-associated antigen. The re-programmed T cells are then expanded *ex vivo* and re-infused back into the same patient.

Proper design of CARs is crucial for eliciting sustained T cell activation in a tumor specific manner. In general, CARs are constructed with two major components—an intracellular T cell signaling domain and the tumor antigen-specific extracellular domain, a single-chain variable fragment (scFv) typically derived from a monoclonal antibody—that are linked via a transmembrane domain to form a fusion chimeric molecule. In essence, a CAR combines the specificity of an antitumor antigen with the downstream T cell effector function and both the intracellular and extracellular domains have implications on the effectiveness of the CAR.

The design of the intracellular component, which promotes the effector function of a CAR, has undergone generational changes (*Figure 1*). First generation CARs have a singular activation domain as its intracellular signaling component, typically the cytoplasmic region of the CD3ζ or Fc receptor γ-chain derived from a T cell receptor. The *in vivo* efficacy of these CARs was ultimately limited due to their failure to maintain persistent T cell activation (15).

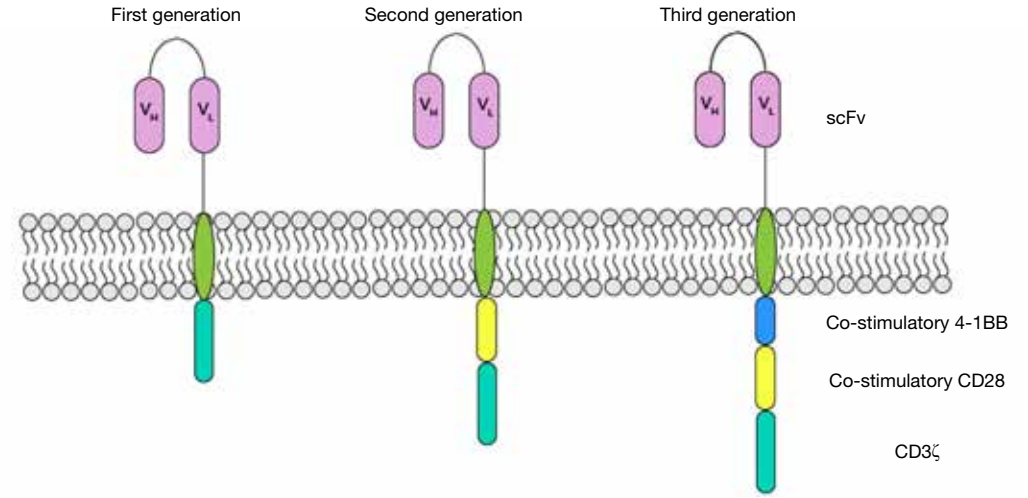

Figure 1 Evolution of chimeric antigen receptors (CARs). First generation CARs were typically engineered with a singular activation domain as its intracellular signaling component (CD3ζ above). Second generation CARs added an additional co-stimulatory domain (CD28 above), while third generation CARs were constructed with multiple co-stimulatory domains (CD28 and 4-1BB above).

This eventually led to the evolution of second generation CARs with an additional co-stimulatory domain (typically CD28, 4-1BB, or OX-40) that increases the expansion and persistence of CAR T cells (16). In third generation CARs, combinations of multiple co-stimulatory domains are added for sustained T cell activation, and while preclinical studies are promising (17-19), early phase clinical trials to test the feasibility of this approach are currently ongoing (20).

The extracellular component of a CAR is of utmost importance to provide the specificity necessary to target a tumor cell. An ideal CAR target is one that is overexpressed on cancer cells, to maximize efficacy, while not expressed on normal tissues, to minimize toxicity. These cancer-specific antigens, however, are rare. In reality, targets are chosen such that they are maximally expressed on tumor cells and minimally expressed on normal tissues to minimize the clinical implications of so-called "off-tumor, on-target" effects. In the B-cell malignancies acute lymphoblastic leukemia, chronic lymphocytic leukemia, and non-Hodgkin lymphoma, CAR T cells directed against CD19 have demonstrated potent, durable activity (10-12,21-23). The result of a CD19-directed CAR is CD19-expressing tumor death and B-cell aplasia, an off-tumor, on-target effect that is effectively managed with intravenous immunoglobulin (11,12). As outlined below, the selection of a target is one of the challenges in the development of CAR therapy in solid tumors given lack of tumor-specific antigens with low potential for clinically significant off-tumor, on-target effects.

Challenges in adapting CAR therapy for solid malignancies

Although early results in various liquid tumors have been promising, several obstacles remain in the application of CAR T cells to solid tumors. While there are challenges to overcome in the manufacturing, expansion, and persistence of CAR T cells inherent to all CAR T cell therapies (24), other key barriers to effective use of CAR T cell therapy in solid tumors include target selection, trafficking of CARs to solid tumors, and the immunosuppressive tumor microenvironment.

Target selection

The selection of a target that maximizes anti-tumor activity with minimal side effects is the holy grail of CAR therapy in solid tumors. One such specific antigen target is epidermal growth factor variant III (EGFRvIII), an immunogenic EGFR variant found only in human tumors such as glioblastoma (25). CAR T cell therapy directed against this variant is currently under investigation (26). Most solid tumors, however, have the potential for significant off-tumor, on-target effects given their lack of specificity in protein expression. Targeting of nonspecific tumor antigens such as carboxy-anhydrase IX and ERBB2 with CAR T cell therapy have demonstrated serious off-tumor, on-target toxicities (27,28).

Potential targets of CAR therapy being evaluated in non-small cell lung cancer or all solid tumors include mesothelin (NCT02580747), MUC1 (NCT02587689, NCT02839954), GPC3 (NCT02876978), CEA (NCT02349724), HER2 (NCT02713984), and EGFR (NCT01869166)(29). Mesothelin, a differentiation antigen with no clearly defined function in normal tissue (30) that is strongly expressed in about 25% of lung adenocarcinoma, particularly in association with a KRAS-mutation (31,32), is an intriguing target; however, it is also expressed in the pericardium, pleura, peritoneum, as well as a number of different tissues throughout the body, albeit at lower levels than that seen in malignant tissues including lung adenocarcinoma (33), raising the concern for potentially dangerous adverse events. In early human clinical trials, mesothelin-directed CAR T cell therapy has been determined to be safe with no off-tumor, on-target toxicities, albeit in small patient numbers (34,35). Each potential target, however, will have potential target-specific adverse events that may limit the universal use of that CAR therapy.

CAR T cell trafficking

In contrast to hematologic malignancies where CAR T cells have exposure to circulating tumor cells bearing the desired target antigen upon infusion, CAR T cells in solid tumors have to migrate to the site of the disease. In many solid tumors, however, the cytolytic effect of the CAR T cell is limited due to restricted T cell infiltration. Trafficking of CAR T cells is controlled by similar mechanisms to normal T cells, namely T cell adhesion, tethering, chemotaxis, and extravasation (36). In solid tumors, each of these processes is dysfunctional. For example, release of angiogenic factors by tumor cells results in formation of new, albeit disorganized and leaky, blood vessels. These angiogenic factors also down-regulate adhesion molecules on the endothelial cells (37). As a result, effector T cells, including theoretically CAR T cells, are unable to efficiently migrate through the blood vessel to interact with the target (38). Moreover, the attraction of cytotoxic T cells to the tumor microenvironment is dependent on interactions between certain chemokines and their appropriate chemokine receptors, including CXCL9/CXCL10 and their receptor CXCR3 or CCL2 and its receptor CCR2. Any imbalance in the interaction between chemokine and chemokine receptor, caused by tumor cells or the associated stroma, limits trafficking of cytotoxic T cells into the tumor microenvironment (24,36). Lastly, the tumor stroma and its fibrosis is a physical barrier to T cell penetration. While non-engineered T cells generally degrade heparin sulfate proteoglycans in the extracellular matrix to penetrate this stroma, *in vitro* cultured T cells lack expression of the key enzyme heparanase, making it harder to penetrate the stroma (39).

Potential interventions to circumvent trafficking problems in CAR T cell therapy include anti-VEGF therapy (40), vasoactive inflammatory cytokines such as tumor necrosis factor (NGR-TNF) to upregulate adhesion molecules and decrease microenvironment hypoxia (41,42), and engineering CAR T cells with chemokine receptors to advance CAR T cell trafficking (43). Although these methods have demonstrated preclinical benefit, their clinical role in making CAR T cell therapy more effective in solid tumors remains to be validated.

Immunosuppressive tumor microenvironment

Once effector T cells are present in the tumor microenvironment, their activity in most solid tumors is hindered by immunosuppressive mechanisms. Within solid tumors, T cell activity can be inhibited by immunosuppressive mechanisms (such as PD-L1, CTLA4, and IDO), depleted amino acids (such as tryptophan and arginine), or depleted oxygen important for T cell survival, and accumulation of immunosuppressive factors such as TGF-β (24,44). Potential avenues to overcome the immunosuppressive environment include the addition of checkpoint inhibitors or VEGF inhibitors to CAR T cell therapy. Interestingly, while IDO overexpression by several tumor types has been shown to diminish the proliferation and cytotoxicity of CD19-directed CAR T cells through induction of apoptosis (45), preconditioning CAR T cell infusion with fludarabine and cyclophosphamide can inhibit IDO expression in solid tumor cell lines (46).

Radiotherapy as an adjunct to immunotherapy

In addition to the aforementioned strategies to overcome barriers to CAR T cell therapy in solid tumors, preclinical evidence supports the use of radiotherapy as an adjunct to engineered T cell therapy to potentially enhance their effectiveness. Radiotherapy, while known for direct local tumor cell death, can also elicit systemic immunomodulatory effects (47). Numerous case reports detail this abscopal effect from radiation therapy, particularly in combination with checkpoint inhibitors (48-50). In addition, several preclinical and clinical studies combining immunotherapy

and radiation have elucidated the mechanism underlying the immunomodulatory effects of radiotherapy, showing response rates of the lesions outside the radiation field from 11% to 25%, and favorable overall survival (51,52).

A recent explosion in clinical trials aimed at evaluating the potential of radiotherapy in conjunction with checkpoint blockade are based on robust systemic responses observed in several preclinical tumor models (53,54). Twyman-Saint Victor *et al*. recently recapitulated the results of a phase 1 trial of ipilimumab and hypofractionated radiation in a melanoma mouse model. Further, they found a resistance signature predictive of response to ipilimumab and radiation therapy. The majority of cells resistant to this treatment had an upregulation of PD-L1, and the combination of radiation therapy, anti-CTLA-4, and anti-PD-L1 therapies resulted in a complete response in 80% of mice. Their results suggested that while anti-CTLA-4 therapy inhibits regulatory T cells, anti-PD-L1 therapy reverses T cell exhaustion, and radiation therapy shapes the T cell receptor repertoire of the expanded peripheral clones (52). Similarly, while the addition of an anti-α-CD137 monoclonal antibody to radiotherapy in triple-negative breast AT-3 model enhanced their response to radiation, it was noncurative until a PD-1 inhibitor was combined with radiation and anti-α-CD137 (55).

Although there is mounting evidence for the systemic immune response elicited by local radiotherapy, particularly in combination with immunomodulatory drugs, there is paucity of data directly examining the potential synergy between radiation and CAR T cell therapy. Nevertheless, a thorough examination of the preclinical data revealing the mechanisms by which radiotherapy elicits a tumor-specific immune response provides a strong rationale for using it to overcome some of the challenges faced by CAR T cell therapy in solid tumors.

Selection of a tumor-specific target for a CAR to minimize off-tumor, on-target effects is dependent on increased expression of the tumor-specific antigen on cancer cells compared to normal tissue. Radiation therapy has the potential to increase the expression of cell surface receptors and tumor-associated antigens. For example, radiation therapy is associated with a dose-dependent increase in the expression of MHC-I molecules on tumor cell surface for several days after completion of treatment (56). Importantly, radiation induces the production of novel proteins that are not present in non-irradiated cells, giving rise to new peptides for recognition by cytotoxic T cells. These results suggest that radiotherapy can enhance the immunogenicity of poorly antigenic tumors, particularly in situations where the absence of a tumor antigen is the gating factor in eliciting a tumor antigen-specific T cell response.

One specific example of how radiotherapy may affect T cell therapy examines CEA-expressing tumors, as CAR T cells directed against CEA are currently under investigation in clinical trials. Preclinical studies demonstrated that radiotherapy directed against CEA-positive M38 cells up-regulated cell surface expression of Fas, a cell surface death receptor that activates a downstream signaling cascade culminating in apoptosis upon binding its ligand FasL (57,58). Moreover, radiation sensitized CEA-positive tumors to CEA-specific T cell killing via the Fas/FasL pathway. Combination of CEA-based vaccine therapy and radiation resulted in significant cures in half of the mice treated, while neither radiation nor vaccine monotherapy achieved significant tumor control. Additionally, there was no response in tumor cells expressing dominant-negative Fas, thus supporting the role of Fas expression in facilitating an anti-tumor immune response (57,58). Similarly, a dose-dependent increase in Fas expression was noted when 23 different human colon, prostate, and lung cancer cell lines were subjected to non-lytic doses of radiation (59). Additionally, there was an increase in the expression of other surface antigens involved in T cell mediated immune responses including MUC-1, CEA, MHC-I, and intercellular adhesion molecule 1 (ICAM-1) in 21 of 23 cell lines. Moreover, five out of the five CEA-positive colon tumor cell lines exhibited significantly enhanced killing by CEA-specific T cells compared to their non-irradiated equivalents. While MUC-1 and CEA overexpression allows antigen-specific targeting of tumor cells, ICAM-1 overexpression has been shown to correlate with increased T cell adhesion and killing (60).

Another example of a target of interest in non-small cell lung cancer in which CAR T cell therapy is currently under study is mesothelin. In mesothelin-expressing xenografts in nude mice treated with a single fraction of 5 or 15 Gy radiation, the mesothelin expression per cell was found to be higher in the radiated group compared to the control group (61), consistent with prior *in vitro* studies (59). Subsequently, half of each group of mesothelin-expressing xenografts were treated with anti-mesothelin immunotoxin SS1(dsFv) PE38 (SS1P), which has a similar scFv as some mesothelin-directed CARs currently under investigation (35). The time to tumor doubling was substantially longer in mice treated with the combination of SS1P and radiation compared to SS1P or radiation alone or the control arm. These results show the ability of radiotherapy to enhance

the efficacy of a mesothelin-specific immunotoxin by increasing the expression of its target antigen on the tumor cell surface (61). Similar increases in c-met and HER2 expression, both targets of interest in non-small cell lung cancer, are noted in the presence of radiation (62,63). While increased tumor antigens may indicate that radiotherapy to one or more malignant lesions can potentially enhance the efficacy of CAR T cell therapy through increased antigenicity of the tumor, or perhaps CAR T cell therapy could make radiation more effective, further evaluation of the combination of CARs and radiotherapy is certainly necessary to make any definitive conclusions.

Other barriers to CAR T cell therapy can also be targeted by radiotherapy. Trafficking of the CAR T cell to the tumor cells is a major issue seen with solid tumors as compared to hematologic malignancies, in part due to a decreased activated T cell adhesion, tethering, chemotaxis, and extravasation, as noted above. Radiotherapy can promote adhesion via up-regulation of adhesion molecules ICAM 1 and VCAM-1 in the tumor microenvironment in an IFN-γ dependent manner (64,65). Furthermore, chemotaxis of activated T cells into the tumor microenvironment can be enhanced by radiation. For example, ionizing radiation increases secretion of CXCL9, CXCL10, and CXCL16, chemokines important for recruitment of activated T cells (66-68). Lastly, radiation can cause remodeling of the tortuous blood vessels within the tumor, allowing for more effective delivery of cytotoxic T cells (66). In fact, remodeling the tumor environment with radiation has been shown to improve adoptive T cell transfer. In a transgenic insulinoma mouse model, the combination of radiotherapy with adoptive cell transfer of tumor-specific activated T cells prompted complete tumor regression, whereas either treatment by itself was ineffective for tumor control (66). This tumor regression was seen in the context of increased chemokine production and remodeling of the capillary network.

Once the cytotoxic T cells are within the often immunosuppressive tumor microenvironment, the role for radiation in improving cytotoxic T cell activity is less clear (69). Whereas some studies in patients undergoing chemoradiation for colon adenocarcinoma demonstrated a reduction of regulatory T cells, a similar effect was not observed in patients with breast cancer (70). Conversely, treatment of colorectal cell lines with high-dose radiation demonstrated reduced regulatory T cells and myeloid derived suppressor cells, and increased CD8+ effector T cells (71). While data is mixed, further evaluation of the effect of radiation as well as dose and fractionation on the immunosuppressive microenvironment is warranted (69,72).

Future perspective

CAR T cell therapy is a promising modality for treating solid tumors, though there are still several issues that need to be resolved to optimize its chance of success. We have outlined some potential roles that radiation may play as an adjunct to CAR T cell therapy. Unfortunately, given the early stages of development of CAR T cell therapy in solid tumors, studies combining it with radiotherapy have not yet been completed.

The preclinical effects noted in the aforementioned trials indicate the immune effects in the radiated lesion. Most CAR T cell therapy trials are done in advanced or metastatic malignancies, so it would be interesting to note whether radiotherapy has a vaccine-like effect on all metastatic lesions. Furthermore, the proper sequencing of radiation and CAR T cell therapy still needs to be determined. Should radiation therapy be given first to kill radiosensitive tumor cells and promote a more immunogenic microenvironment, in turn allowing CAR T cells to more easily penetrate the tumor and target cells with overexpressed antigens (73)?

Additionally, what is the optimal dose and fractionation of radiation? In order to answer, further efforts need to sort out mixed data presented on the effect of radiotherapy on the immunosuppressive tumor microenvironment. One study indicated that a high dose of radiation was enough to alter the immunosuppressive environment, but small doses over several fractions were ineffective. Furthermore, as there is an increase in number of clinical trials evaluating the combination of radiotherapy and checkpoint inhibitors, a trial combining radiotherapy, CAR T cell therapy, and checkpoint inhibitors to minimize the immunosuppressive environment would be interesting. This could follow from planned studies evaluating CAR T cell therapy and checkpoint inhibitors.

Particular focus will be required to carefully evaluate safety and develop ways to minimize or ameliorate serious unexpected toxicities. For example, the ROCKET trial, a phase 2 study of an anti-CD19 CAR T cell therapy JCAR015 in adults with relapsed or refractory acute lymphoblastic leukemia (NCT 02535364) was recently halted by the FDA due to multiple patient deaths (74), as a result of neurotoxicity likely secondary to fludarabine preconditioning. Given the immunomodulatory benefits of preconditioning regimens (75), one possibility that could be explored in future trials is use of radiation as a potential substitute to overcome the

immunoinhibitory microenvironment.

The role of CAR T cell therapy in solid malignancies in combination with radiation therapy is unknown but remains promising. Clinical trials evaluating the feasibility of this approach are about to begin, such as a phase 1 study at Duke University (NCT02664363) which aims to evaluate the safety and efficacy of EGFRvIII CAR T cells in combination with the standard of care radiation therapy and concurrent temozolomide in newly diagnosed glioblastoma patients. Continued advancements in pairing CAR T cell therapy with other therapies such as radiation will help to advance its development.

Conclusions

CAR T cell therapy is a promising emerging tool for the treatment of solid tumors. However, minimizing normal tissue toxicity by finding appropriate tumor targets, optimizing delivery of CAR T cells to tumors, and overcoming the immunoinhibitory tumor microenvironment are some of the barriers to overcome prior to mainstream use of this promising therapy. Radiation therapy has the potential to overcome some of these challenges as it has been shown in preclinical studies to increase the expression of various tumor antigens as well as play a crucial role in chemotaxis and counteracting the inhibitory tumor microenvironment. While yet to be explored in depth, there is strong preclinical rationale for combining radiation therapy and CAR T cells in future experiments to explore the synergistic effects between these two modalities.

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Footnote

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Clinical experiences of combining immunotherapy and radiation therapy in non-small cell lung cancer: lessons from melanoma

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Abstract: Radiation therapy (RT) is an essential component of local control for non-small cell lung cancer (NSCLC), but distant failures dictate the poor prognosis of this disease. Until recently, the possibility of using RT as an immunoadjuvant to stimulate a systemic anti-tumor immune response was not a realistic clinical opportunity. The emergence of immune checkpoint blockade as an effective immunotherapy for NSCLC has opened the door for combinatorial approaches involving RT. In melanoma, the body of preclinical evidence combining radiation and immunotherapy buoyed clinical efforts, from which promising results have begun to emerge. Preclinical work combining radiation and immunotherapy indicate similar findings in NSCLC, and clinical efforts are ongoing. Here, we review the rationale, preclinical evidence, ongoing efforts and anticipated challenges of efforts combining radiation and immunotherapy in NSCLC.

Keywords: Radiation; immunotherapy; checkpoint blockade; non-small cell lung cancer (NSCLC); lung cancer

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Introduction

The emergence of immunotherapy as the fourth pillar of cancer therapy, alongside surgery, radiation and chemotherapy, has generated interest in combinatorial strategies to maximize the benefits of anti-tumor immunity. The Food and Drug Administration (FDA) approval of immune checkpoint blockade in the treatment of metastatic non-small cell lung cancer (NSCLC) has specifically ignited efforts toward combinatorial approaches in NSCLC. Radiation therapy (RT) may be well suited as a partner to immunotherapy in NSCLC given its immunoadjuvant properties and its utility across the spectrum of patients with NSCLC. While combinations of RT and immunotherapy in NSCLC are still nascent, there is a body of preclinical literature supporting this approach. Moreover, emerging clinical data in melanoma and other disease sites support testing combinations of RT and immunotherapy in NSCLC.

The need for greater systemic control in NSCLC

NSCLC is the leading cause of cancer death in the United States with an estimated 158,080 deaths in 2016 (1). RT plays a prominent role in the treatment of NSCLC patients across the spectrum of disease: early stage, locally advanced and metastatic. In the growing population of patients with early stage disease, curative local therapy (surgery or stereotactic body radiotherapy) results in 5-year survival rates below 55% (SEER 2005–2011) and 3-year distant metastasis rates between 20–40% (2). For patients with locally advanced or metastatic disease, outcomes remain poor: 5-year survival rates reach 25% for locally advanced stage IIIA patients (2). Patients with metastatic disease

treated with platinum-doublet therapy have a median survival less than 1 year.

In the absence of better systemic disease control, even the most effective RT will have limited gains. Cytotoxic chemotherapy has had limited efficacy for patients with locally advanced and metastatic NSCLC. Several studies demonstrated a survival benefit to adjuvant chemotherapy after resection of non-metastatic NSCLC (stage II-III) (3-7). Adjuvant chemotherapy does not improve clinical outcomes in stage IA patients. For stage IB patients, CALGB 9633 found no benefit to adjuvant chemotherapy, notwithstanding a posthoc analysis suggesting benefit for patients with tumors >4.0 cm (8). Even in the unplanned subgroup analysis of patients with tumors >4.0 cm, the modest benefit of adjuvant chemotherapy must be weighed against its toxicities.

Other approaches, including angiogenesis inhibitors, have had limited success (9,10). The emergence of next generation sequencing has helped identify patients whose tumors harbor mutations for which targeted therapies exist. With this type of personalized medicine, profound responses are observed. However, this approach is limited to a small subset of patients whose tumors harbor actionable mutations (11).

Thus, additional approaches are needed to combat this devastating disease. Immunotherapeutic approaches are the most promising of the emerging therapies for NSCLC. These approaches were first successful in melanoma, a disease in which the immune activating factor IL-2 emerged as a standard therapy in the 1980s (12). Adoptive cell transfer of tumor infiltrating lymphocytes (TIL), and subsequently T cell receptor engineered lymphocytes, induced profound responses in melanoma in the 1990s and early 2000s. But it was not until 2011, when level I evidence demonstrated the survival benefit of immune checkpoint blockade targeting the immunoregulatory molecule CTLA-4, that immunotherapy attracted the attention of a global audience (13). This was followed closely by clinical trials demonstrating the efficacy of a second class of immune checkpoint inhibitors targeting programmed cell death protein 1 (PD-1) (14).

In 2015, immune checkpoint inhibitors targeting the PD-1 pathway were approved by the U.S. FDA for treatment of patients with locally advanced or metastatic NSCLC that had progressed on prior therapy. More recently the results of the KEYNOTE-024 trial randomized previously untreated patients with metastatic PD-L1 positive NSCLC to pembrolizumab, a humanized monoclonal antibody against PD-1, versus standard chemotherapy and showed a significant survival benefit in favor of patients receiving pembrolizumab (15).

Rationale for immunotherapy in NSCLC: the shoe fits

Before the approval of immune checkpoint blockade (anti-PD-1 therapy) for metastatic NSCLC, there were hints in studies of the immune microenvironment of these tumors that immunotherapy could be an effective approach. Several studies have examined the association of tumor infiltrating lymphocytes TIL on outcome in patients with NSCLC. The interest in TIL may be largely based on observations of heavy T-cell infiltrates in melanoma, a disease highly responsive to immunotherapy. In melanoma, the rich T-cell infiltrate serves as the basis for TIL therapy, in which TIL are grown *ex vivo* for therapeutic intravenous administration in combination with myeloablative regimens and immune growth factors (e.g., IL-2).

Across numerous studies, the presence of CD8+ T cells within the NSCLC tumor microenvironment was associated with superior clinical outcome, despite heterogeneous clinical populations and quantification methods (16). Even more recently, a study examined the association of TIL with survival in a more homogeneous population of patients with resected NSCLC who were enrolled on clinical trials testing adjuvant chemotherapy. The presence of TIL was associated with improved overall survival in this dataset (17). These data supporting TIL as a prognostic factor lend credence to the potential anti-tumor role of infiltrating T cells in NSCLC, as in melanoma.

In addition to TIL, the burden of somatic mutations in a particular tumor may determine its immunogenicity. Effective immunotherapy is based on the premise that T cells recognize a non-self antigen presented by tumor cells. One hypothesis is that tumors with greater mutational load also have greater neoantigens repertoire, increasing the likelihood of a productive anti-tumor T cell response. Compared to other cancer types, melanoma has the highest burden of somatic mutations, thought to be a result of UV-light exposure (18). Consistent with this hypothesis, melanoma patients with higher mutational load are more likely to derive clinical benefit from anti-CTLA-4 immune checkpoint blockade (19).

The two major subtypes of NSCLC, squamous cell carcinoma and adenocarcinoma, carry the second and third highest mutational burden of all cancer types. This

heavy mutational signature may be related to the effects of tobacco smoking. It is plausible that like melanoma, the greater mutational burden seen in NSCLC results in a higher likelihood of tumor reactive T cells that may mount a productive anti-tumor response under the appropriate stimulus. And in line with findings in melanoma, NSCLC patients with tumors harboring higher mutational load, anti-PD-1 immune checkpoint blockade was more effective (20).

Clinical data for immunotherapy in NSCLC

The FDA approval of pembrolizumab was based on results of an international phase 1 study of pembrolizumab (KEYNOTE-001) that included 495 patients with locally advanced or metastatic NSCLC (including both squamous and non-squamous histology). In these patients there was an overall response rate of 19.4%, including a response rate of 45.2% in the PD-L1 high-expressing population (21). Because of the latter finding, its approval was limited to patients with tumors expressing PD-L1.

Two trials compared an alternate antibody antagonist of PD-1, nivolumab, with docetaxel in the second-line treatment of metastatic squamous or non-squamous lung cancer (CheckMate-017 and CheckMate-057, respectively) (22,23). For both CheckMate-017 and CheckMate-057, 2-year overall survival was higher in the nivolumab arm (23% *vs*. 8% and 29% *vs*. 16%, respectively). Unlike CheckMate-057, there was a statistically significant increase in progression-free survival for patients on CheckMate-017 and responses were not tied to baseline PD-L1 expression. Nivolumab was approved by the FDA in March of 2015 for treatment of advanced squamous cell NSCLC refractory to chemotherapy.

Despite the overwhelming success of immune checkpoint blockade, the majority of patients do not respond. Combination therapies—including those with RT—may circumvent the resistance mechanism and expand the efficacy of immune checkpoint blockade to larger fraction of patients with NSCLC.

Rationale for radiotherapy as an immunoadjuvant

Radiation is classically categorized as a DNA-damaging, cytotoxic therapy. However, in addition, it acts as an immunomodulator. The interest in radiation as an immunomodulator began nearly half a century ago, with the first anecdotal reports of the abscopal effect (24). The abscopal effect is a clinical response in a malignant lesion other than the target lesion (or a response outside the irradiated field), and was posited to be a result of an immunologic response incited by radiation to the target lesion.

Over the subsequent decades, scattered cases of the abscopal effect were reported, but these were met with healthy skepticism. As immune checkpoint blockade and other immunotherapies began to emerge over the past decade, reports of the abscopal effect became more prominent (25). Concurrently, the body of preclinical evidence on the intersection of radiation and immunity began to grow exponentially. These have been reviewed extensively elsewhere (26-28).

Importantly, the local effects of radiation were noted to be immune dependent in specific mouse models. In a murine model of melanoma, the depletion of CD8+ T cells abrogated the local effects of irradiation (29). Likewise, in a murine model of colon cancer, the local effects of radiotherapy were partially dependent on IFN-alpha receptor and stimulator of interferon genes (STING) signaling (30). In both of these studies, the local anti-tumor effects of radiation could not be uncoupled from the T cell immunity. That radiation can incite a local anti-tumor T cell response supports the possibility that it may incite a systemic anti-tumor T cell response, otherwise known as concomitant immunity (31).

Indeed, studies in an animal model of breast cancer demonstrated that ablative radiation of a local tumor impeded the development of lung metastases in a CD8+ T-cell dependent fashion (32). These results have been corroborated by several studies where local irradiation and systemic immunotherapy led to improved tumor control compared to either therapy alone (32-36).

Several mechanisms have been proposed by which radiation potentiates an anti-tumor T cell response. Radiation can induce the release of danger signals or danger-associated molecular patterns (DAMPs), which serve as the first step in a cascade that leads to activation of antigen presenting cells (APCs)/dendritic cells. Danger signals, such as HMGB1, can bind to toll like receptors on the surface of APCs and potentiate the ability of APCs to activate nearby T cells (37). Thus, indirectly via release of DAMPs or via release of specific chemokines (e.g., GM-CSF), radiation may modulate antigen presentation. Radiation can also lead to increase in the quantity, variety and presentation of antigens from a tumor though activation of proteasome pathways, and augmentation of MHC class I presentation (38,39). Radiation-induced chemokines may

also directly attract APCs or effector CD8+ T cells to the tumor microenvironment (40).

Radiation can also have immunosuppressive effects that may counteract the development of systemic anti-tumor immunity. Foremost, radiation can recruit immunosuppressive myeloid cells (e.g., myeloid derived suppressor cells, inflammatory monocytes, or tumor-associated macrophages) that either directly promote tumor outgrowth (41), or contribute to be an immunosuppressive microenvironment in which T cells are dysfunctional (42). Additionally, radiation has been shown in pre-clinical models to upregulate PD-L1 expression in the tumor microenvironment, which can lead to T-cell exhaustion (36). Finally, radiation can lead to accumulation of T-regulatory cells, which serve as obstacles to productive anti-tumor immunity (43).

Whether the dominant effects of radiation promote or disrupt anti-tumor immunity may largely depend on tumor type and context. In tumors with baseline immunogenicity, radiation may be more likely to stimulate productive anti-tumor immunity. On the contrary, immunologically "cold" tumors that lack a neoantigens signature and T cell infiltrate, may predominantly recruit immunosuppressive myeloid cells in response to radiation.

Abscopal response to radiotherapy in NSCLC

The majority of clinical data indicating an immunoadjuvant role for radiation are from patients with melanoma. However, data in NSCLC have also emerged. In 2013, clinicians reported a case of an abscopal response to radiotherapy in a patient with metastatic NSCLC who did not receive systemic therapy. The patient received conventionally fractionated radiation (60 Gy) for a T3N0 left upper lobe primary adenocarcinoma, and SBRT (26 Gy ×1) to a right lower lobe primary adenocarcinoma. Two weeks after treatment the patient was noted to have an FDG avid lesion in the adrenal gland consistent with metastasis, and approximately 2 months after treatment developed an FDG avid humeral lesion, also consistent with metastasis. One year after radiation, even without systemic therapy, these lesions had achieved complete metabolic response. The patient ultimately progressed at a different osseous site (44).

The potential for radiation to initiate a systemic anti-tumor immune response has been used as an argument for the treatment of early stage NSCLC with SBRT, especially in contrast to surgery. In a 2010 retrospective study comparing patients with T1-T2N0 NSCLC who underwent either wedge resection or SBRT, local and locoregional recurrence rates were lower in patients receiving SBRT (45). However, in the pooled analysis of two randomized trials of surgery versus SBRT in medically operable patients (STARS and ROSEL), while SBRT was as effective as surgery (46), there was no significant difference in the rate of regional recurrence or distant metastasis.

Radiotherapy and immune checkpoint blockade

The ability of radiation to initiate systemic anti-tumor immunity may be amplified in the context of immune checkpoint blockade. In animal models, radiation interacts favorably with immune checkpoint blockade. When combined with dual immune checkpoint blockade in a murine model of melanoma, radiation is associated with T cell receptor diversification, and results in greater control of non-irradiated tumors (47). A concordant phenomenon was seen in the peripheral blood from a cohort of patients with metastatic melanoma treated with immune checkpoint blockade (anti-CTLA-4) and palliative radiotherapy. In this cohort, 17% of patients experienced a response in the non-irradiated lesion, which exceeds the expected response rate for anti-CTLA-4 therapy alone.

In the largest dataset thus far examining the effects of local therapy in combination with immune checkpoint blockade (anti-CTLA-4), 127 metastatic melanoma patients were treated with electrochemotherapy, radiation or selective internal radiation therapy (SIRT). After accounting for measured differences in the treatment groups, patients receiving local therapy had significantly longer overall survival (48).

In NSCLC, there are case reports of abscopal responses to radiotherapy in patients also receiving immune checkpoint blockade. In one case, a patient with metastatic lung adenocarcinoma who had progressed despite multiple systemic therapy regimens, received RT concurrently with anti-CTLA-4 therapy and experienced a clinical response in multiple metastatic lesions (49). While it is possible this response was primarily related to anti-CTLA-4, it should be noted that anti-CTLA-4 therapy alone or in combination with systemic therapy has not resulted in improvements in disease outcomes for patients with NSCLC.

A recently reported phase I study of anti-CTLA-4 therapy with SBRT in patients with non-melanoma solid tumors included eight patients with NSCLC (50). Uniquely in this trial, patients received ablative doses of radiation, with BED of ~100 Gy (assuming alpha/beta =10) to the

lung or liver. One out of eight NSCLC patients experienced either a partial response or prolonged (6 months) stable disease outside of the irradiated field (according to immune related response criteria).

Toxicity considerations

Multiple early phase studies have been conducted examining the safety of combining ablative radiotherapy with immunotherapeutic agents (47,51,52). At the University of Pennsylvania, combination ipilimumab with hypofractionated palliative radiotherapy was tested in a phase I study. Of 21 enrolled patients, there was no grade 4 or higher toxicities. The most common grade 3 toxicity was anemia, which was unlikely to be related to the effects of focal hypofractionated radiotherapy.

For the combination of lung radiation and immune checkpoint blockade, pneumonitis is an overlapping toxicity. Radiation pneumonitis and pneumonitis due to immune checkpoint blockade may have shared mechanisms. The precise mechanism for radiation pneumonitis is unclear, but in addition to innate immune responses driven by monocyte, macrophage and neutrophil infiltration (53,54), T cell driven adaptive immune processes have been implicated (55-57). The mechanism for pneumonitis related to immune checkpoint blockade has not been well studied, but is putatively due to autoreactive T cells.

Anti-PD-1 agents have a better safety profile than anti-CTLA4 agents. The rate of grade 3 toxicity for pembrolizumab in NSCLC in KEYNOTE-001 was <10%, including a 1.8% rate of grade 3 pneumonitis. And in modern SBRT series, the rate of grade 3 radiation pneumonitis is <5% (58).

Thus, given the low independent rates of pneumonitis with each therapy, this combination will likely be safe and tolerable. However, given potential overlapping immune mechanisms of pneumonitis related to each therapy, it is important to carefully characterize the safety and toxicity profile in a prospective study. In the aforementioned phase I study of anti-CTLA-4 therapy with SBRT in patients with non-melanoma solid tumors, which included eight patients with NSCLC, clinical pneumonitis was not observed. A phase I safety study of atezolizumab (anti-PD-L1 antibody) in combination with SBRT in early stage NSCLC is actively recruiting patients (NCT02599454, www.clinicaltrials.gov). Likewise, in the metastatic setting, escalating doses of radiotherapy to the lung will be investigated combination with pembrolizumab to assess safety and toxicity (NCT02587455).

Ongoing studies

In addition to the phase I study of SBRT combined with anti-PD-L1 in early stage NSCLC, other studies are examining combinations of immunotherapy and radiation or chemoradiation in NSCLC (*Table 1*). Many of the trials are conducted in the metastatic or oligometastatic setting, similar to those described above, wherein patients with NSCLC are included with other histologies and the emphasis is to examine the impact of SBRT on efficacy of immune checkpoint blockade or other immunotherapy. At one institution, investigators are testing FLT3 ligand, which may enhance antigen presentation, administered subcutaneously concurrent with SBRT in patients with metastatic NSCLC refractory to standard therapy (NCT02839265).

In studies of early stage and locally advanced disease, the focus of combination studies is to test the efficacy of immunotherapy when added to standard therapy. This includes immunotherapy other than immune checkpoint blockade, such as cancer vaccines that have been tested in the metastatic setting (e.g., cancer-testis antigens such as NY-ESO-1 and MAGE-A3, as well as telomerase and MUC-1). A phase II study of telomerase peptide vaccination in locally-advanced stage IIIA NSCLC suggested patients developed specific immune responses against the peptide in 80% of patients (59). Another vaccine study in locally advanced patients, this one a phase III randomized study, examined the impact of a vaccine against MUC1 glycoprotein on overall survival. While no overall survival difference was noted, the vaccine was associated with a benefit in a subset of patients who had received prior chemoradiotherapy (60).

Other vaccine studies are ongoing for patients after chemoradiation for locally advanced disease. However, combinations using immune checkpoint blockade have generated the most interest, and several trials are ongoing for locally advanced patients. RTOG will be studying adjuvant nivolumab after definitive chemoradiation for locally advanced NSCLC in a randomized phase III design (NCT02768558). Another institution is testing the use of neoadjuvant pembrolizumab for patients with stage IB, II or IIIA NSCLC in a single arm phase II design (NCT02818920). Durvalumab, an anti-PD-L1 monoclonal antibody, will be tested as a neoadjuvant therapy in combination with chemotherapy in patients with resectable stage IIIA NSCLC

Table 1 Ongoing clinical trials combining radiation and immunotherapy for NSCLC

Institution	Disease	Design	Primary outcome	Immunotherapy	Radiation	Combination timing
UC Davis	Stage I NSCLC	Single arm phase I	Safety	Atezolizumab (anti-PD-L1)	SBRT 50 Gy	Concurrent
Weill Medical College of Cornell Univ.	Stage IB and II NSCLC	Randomized phase II	DFS	Durvalumab (anti-PD-L1)	Randomized to surgery or preop SBRT	Concurrent
RTOG/Multi-Institutional	Stage III NSCLC (unresectable)	Phase III randomized	OS, PFS	Nivolumab (anti-PD-1)	Thoracic chemoradiation 60 Gy	Immunotherapy after chemo-radiation
Duke Univ./Multi-Institutional	Stage IB, II or IIIA NSCLC	Single arm phase II	Surgical feasibility	Pembrolizumab	Adjuvant thoracic RT if necessary	Not concurrent
UC Davis	Stage IV or recurrent NSCLC	Single arm phase I	safety	MPDL3280A (anti-PD-L1)	SBRT	Concurrent; induction; or sequential
Royal Marsden Hospital	Metastatic NSCLC	Phase I dose escalation	safety	Pembrolizumab (anti-PD-1)	Palliative RT (low-dose, high-dose)	
Albert Einstein College of Medicine	Metastatic NSCLC	Phase 2	PFS	FLT3 ligand (CDX-301)—given subcutaneously	SBRT to lung lesion	Concurrent

NSCLC, non-small cell lung cancer; DFS, disease-free survival; PFS, progression-free survival; OS, overall survival; SBRT, stereotactic body radiotherapy.

(NCT02572843). Patients will also receive adjuvant durvalumab, but only after adjuvant radiotherapy, should it be indicated. While these studies may prove a benefit for the experimental agent, they are unlikely to produce data on the potential synergy and radiotherapy.

Resistance mechanisms/future directions

While optimism is abounding regarding these combinatorial successes, failure of these approaches can and should be anticipated. In melanoma, where immune checkpoint blockade has had startling success, a substantial number of refractory cases remain. Some patients do not respond to therapy, and others respond and then progress. Recent evidence suggests that defects in interferon sensitivity within tumor cells may limit the efficacy of immune checkpoint blockade (61,62). Interferon pathway stimulation is likely an important component of the radiotherapy-induced antitumor response as well (30). Thus, identifying patients with NSCLC that lack sufficient interferon pathway signaling, will allow for better selection of patients for clinical trials of combined radiation and immunotherapy.

Conclusions

Some of the immune effects of radiation can induce or boost systemic anti-tumor immunity, especially in tumor with baseline immunogenicity. By doing so, radiation can complement immunotherapies such as immune checkpoint blockade. This is an especially promising approach in NSCLC because of its shared features with melanoma, the disease where immunotherapy has made the most headway. Like melanoma, NSCLC is characterized by a significant number of somatic mutations and a substantial T cell infiltrate. Given these similarities, it is not surprising that NSCLC is one of a handful of malignancies that, like melanoma, responds to immune checkpoint blockade. With the emerging data in melanoma that radiotherapy may improve clinical response rates and outcomes with immune checkpoint blockade, this approach is equally intriguing in NSCLC. We await results from numerous studies testing this approach in the early stage, locally advanced and metastatic settings.

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Footnote

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Fractionated radiotherapy combined with PD-1 pathway blockade promotes CD8 T cell-mediated tumor clearance for the treatment of advanced malignancies

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Programmed death-1 (PD-1) is a T cell inhibitory receptor, expressed on recently activated and chronically stimulated CD4 and CD8 T cells (1,2). Through interacting with programmed death ligand-1 (PD-L1), PD-1 limits T cell receptor signaling, and maintains peripheral tolerance (1,2). PD-1 pathway blockade has the potential to restore effector function to exhausted T cells, thus boosting their antiviral and antitumor activity (2). This has prompted the development of PD-1/PD-L1 antibodies for treating cancer. Success in numerous preclinical studies (3-5) led to multicenter clinical trials, and FDA approval of anti-PD-1 agents (nivolumab or Opdivo® and pembrolizumab or Keytruda®) for the treatment of metastatic melanoma and non-small cell lung cancer (6-8). With as many as 31% of patients benefiting from treatment and median response duration lasting 2 years (8), it is not surprising that PD-1 pathway blockade, and interference with other T cell signaling checkpoints such as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), continues to generate excitement for cancer immunotherapy. Given that PD-1 and CTLA-4 blockade exert distinct effects on tumor control, combined blockade has been successful in mouse models of melanoma and clinical trials with 53% response rate (9). In order to further increase efficacy, it is necessary to understand how immunotherapy could complement already approved treatment modalities, such as chemotherapy and radiation.

Radiation therapy (RT) is the most widely used non-surgical intervention for treating primary solid malignancies, and relieving cancer-associated pain stemming from bone metastases (10). Besides directly inducing tumor cell death, RT has an immunomodulatory effect. Dying tumor cells release danger-associated molecular patterns (DAMPs) such as deoxyribonucleic acid (DNA), high mobility group box 1 (HMGB-1) and adenosine triphosphate (ATP), as well as tumor-associated antigens. DAMP recognition induces a strong type I interferon (IFN) signature in dendritic cells, increases major histocompatibility complex (MHC) Class I and Class II expression, and helps prime tumor-reactive CD4 and CD8 T cells. Immune-mediated regression of the irradiated tumor and improved control of non-irradiated lesions (abscopal effect) sometimes arise in RT-treated hosts (10). However, more often than not, RT is not sufficient to override tumor-induced immunosuppression and escape (10). Several case reports pointed to a potential benefit of combining RT with immune checkpoint intervention, while a retrospective study and a phase I and II trial concluded combination therapy was safe (11-13). This has spurred extensive preclinical investigation into RT-checkpoint blockade combination therapies, with a focus on dosing, scheduling, and mechanisms underlying potential synergistic effects. RT delivered in smaller daily fractions over several days increases tumor immunogenicity compared to single-dose radiation (14). Previous work has shown that fractionated RT synergized with CTLA-4 blockade as well as other immunotherapies (e.g., Toll-like receptor 7 agonist treatment) to yield better survival than single-dose radiation, or single-dose radiation with immunotherapy (15,16).

Recently, Dovedi and colleagues published an exciting report in *Cancer Research* examining whether fractionated RT combined with PD-1 pathway inhibition could enhance

survival following tumor challenge (17). The authors first noted that fractionated RT (delivered in 5 daily fractions of 2 Gy) led to increased PD-L1 expression on CT26 colon carcinoma cells. Interestingly, this effect was noted *in vivo* and not *in vitro*, suggesting that tumor-associated stroma or infiltrating T cells mediated the increase in PD-L1 expression after RT. Through elegant depletion experiments, the authors demonstrated that CD8 T cells were required for the enhanced PD-L1 expression on the tumor cells. Since IFNγ can induce PD-L1 expression (18), the authors utilized anti-IFNγ neutralizing antibodies and IFNγR1 shRNA to determine whether the noted increase in PD-L1 expression was IFNγ-dependent. Blocking IFNγ signaling abrogated RT-induced PD-L1 expression in the presence of CD8 T cells, suggesting that CD8 T cell-mediated IFNγ secretion is responsible for enhanced PD-L1 expression on CT26 cells (17). The increased PD-L1 expression following RT could therefore explain how some tumors evade the endogenous immune response, and provides a rationale for combining checkpoint blockade with RT for enhanced tumor control. To test the functional significance of RT-induced PD-L1 increase, Dovedi *et al.* combined fractionated RT with PD-1 pathway blockade. Mice bearing established CT26, 4T1 (triple negative breast) or 4,434 (melanoma) tumors exhibited significantly improved tumor control (184.3±13.5 *vs.* 292.8±14.3 mm^2 for 4T1 at day 10 post treatment) and overall survival when treated with fractionated RT and either anti-PD-1 or anti-PD-L1, than the animals treated with either monotherapy (17). Specifically, 66–80% of treated animals survived past 100 days and were protected from a subsequent recall challenge at a distinct site (17).

CD8 T cells were critical for this tumor control, as CD8 T cell depletion prior to therapy abrogated the protective effect of combined treatment (17). NK cell depletion impacted initial tumor growth, but not overall survival (17). Collectively, these findings suggest that CD8 T, but not NK cells, are necessary and sufficient for tumor control after RT and PD-1 pathway blockade. Dovedi and colleagues also depleted CD4 T cells prior to RT + anti-PD-L1 treatment, and noticed improved tumor control, albeit without a significant increase in survival (17). These data indicate that regulatory T cells (T_{regs}) might play a role in restraining antitumor immunity after combined therapy. To test this, future studies could incorporate targeted T_{reg} depletion (e.g., using Foxp3-DTR mice) or anti-CTLA-4 treatment.

The authors noted that the combination therapy was well tolerated, since body weight was not impacted (17). However, with the use of checkpoint blockade, emergence of autoimmune-like events or overt autoimmunity is a major concern. Specifically, 9–14% of patients treated with anti-PD-1 or anti-PD-L1 developed immune-mediated grade 3 or 4 adverse events, which affected the skin, gastrointestinal tract, or thyroid (6,7). In addition, several patients developed type 1 diabetes as a result of PD-1 pathway inhibition (6,19). Future studies could evaluate serum autoantibody levels, intestinal pathology, and kidney and liver toxicity as direct readouts of immune-related adverse events following combination therapy.

Translating combination therapy into the clinic requires optimizing treatment schedule for maximizing clinical benefit, while minimizing side effects. In their report, Dovedi *et al.* investigated whether the order in which RT and PD-1 pathway blockade were administered affected treatment efficacy. Starting anti-PD-L1 treatment on the first or the last day of fractionated radiotherapy cured 57–60% of treated animals (17). However, anti-PD-L1 administered 7 days after the last dose of radiotherapy had no additive effect compared to radiation alone, and yielded no long-term survivors (17). These findings suggest that checkpoint blockade is most effective during, but not following radiation, and warrants further investigation.

In the year since Dovedi *et al.* published their findings, a phase I clinical trial examined the benefit of fractionated radiotherapy and CTLA-4 blockade in 22 patients with stage IV melanoma (20). Patients received fractionated RT, followed by four cycles of ipilimumab (anti-CTLA-4) treatment and were monitored for response with computed tomography (CT). Partial response (at least a 30% decrease in lesion diameter) was noted in 18% of patients (20). Another 18% had stable disease, while 64% of treated patients experienced progressive disease, suggesting that the majority of patients did not respond (20). Twyman-Saint Victor *et al.* then applied this treatment regimen to mice bearing B16-F10 melanoma, and similarly to Dovedi and colleagues, noted that concurrent checkpoint blockade synergized with RT, in a CD8 T cell-dependent manner. However, only 17% of animals responded to treatment. Even though combined treatment decreased the number of T_{regs} in the tumor, the number of effector CD8 T cells failed to increase (20). Importantly, transcriptional analyses of resistant tumors revealed that PD-L1 was in the top 0.2% of up-regulated genes that make up the gene signature of tumors refractory to combination therapy (20). Genetic deletion of PD-L1 by CRISPR rendered a resistant tumor cell line highly responsive to RT + anti-CTLA-4 therapy.

This prompted the authors to treat B16-F10 tumor bearing mice with anti-PD-1/PD-L1 in addition to anti-CTLA-4 and fractionated RT. In this case, 80% of animals were long-term survivors, with protective immunity against subsequent challenge (20). The authors showed that the three treatment modalities evoked non-redundant immune mechanisms. Radiotherapy led to increased CD8 T cell diversity in the tumor, CTLA-4 blockade decreased the number of tumor-infiltrating T_{regs}, while PD-L1 blockade allowed reinvigoration of exhausted intratumoral CD8 T cells (20).

Checkpoint blockade has revolutionized cancer therapy, and given hope to patient populations suffering from standard treatment-refractory tumors. Further understanding the ways in which checkpoint inhibitors complement each other and synergize with other therapies is necessary for increasing objective responses, minimizing relapse and side effects. Preclinical studies have already begun to explore optimal treatment schedules, and understand pathways driving resistance to combination therapy (17,20). Future work should focus on identifying biomarkers to predict treatment efficacy, as well as autoimmune risk screening (e.g., HLA typing, autoantibodies) to identify patients likely to develop immune-related adverse events.

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Footnote

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Immunotherapy and radiation therapy for operable early stage and locally advanced non-small cell lung cancer

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Abstract: Non-small cell lung cancer (NSCLC) is the most common cause of cancer mortality. Although a significant proportion of patients can be cured with surgery, with or without adjuvant or neoadjuvant chemotherapy and radiation, a significant proportion of patients will fail, particularly distantly. Over fifty percent of patients present with stage IV disease. There are multiple forms of immunotherapy available including T-cell transfer, cytokine therapy, and oncolytic viruses. Checkpoint inhibitors have shown tremendous activity in NSCLC and are currently under intense study given promising data on response. Immunotherapy and radiation therapy (RT) both show significant immune editing activity in NSCLC that may allow the innate and adaptive immune system to help control systemic disease by both radiosensitization and a sustained systemic immune response. Multiple clinical trials are underway exploring the role of adjuvant or neoadjuvant immunotherapy in operable NSCLC. A substantial amount of progress is to be made in terms of optimizing radiation dose and fractionation, immunotherapy type and dose, and integrating both to best realize the benefits of immunotherapy and radiation in operable lung cancer.

Keywords: Radiation therapy (RT); immunotherapy; non-small cell lung cancer (NSCLC)

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Introduction

Lung cancer is the leading cause of cancer mortality in the United States. About 25% of patients with non-small cell lung cancer (NSCLC) present with early stage disease, which is potentially curable with standard of care lobectomy (1,2). Local control is generally excellent after surgery or radiation. Stereotactic body radiotherapy (SBRT) has become an excellent alternative treatment option in patients with early-stage, node negative disease (3). Local control is about 90% at 3 years. Early data in patients with operable patients treated with SBRT indicate local control is 92% and 73% at 5 years for T1 and T2 disease, respectively (4).

For patients with node-positive or locally advanced operable disease, conventionally fractionated radiation therapy (RT) can be integrated in several different ways. In resectable locally advanced patients, typically stage IIIA (AJCC v7), primary surgery is performed before or after platinum-based chemotherapy, and post-operative RT is indicated in disease with persistent N2 lymph nodes. Preoperative chemoradiotherapy is an alternative approach to the treatment of resectable N2 disease. Patients with unresectable locally advanced NSCLC are treated with curative intent concurrent or sequential chemoradiation.

However, there is a critical need to develop better therapeutic approaches to treat patients with early and locally advanced stage disease and to integrate systemic therapies that have the capacity to effectively eradicate micrometastatic disease and create a sustained systemic response.

Patients with early stage disease still have high risk of relapse

Although local control is high for patients with operable NSCLC, systemic relapse remains the predominant failure pattern. Even among patients with the earliest clinical stage of lung cancer, 50% will die within 5 years of diagnosis after lobectomy (5). For patients with Stage II and IIIA disease and good performance status, platinum-based chemotherapy is recommended to improve systemic relapse rates.

There exists a subset of early stage patients with identifiable poor prognostic characteristics. This includes patients with a suboptimal gene profile, and a number of variably expressed tumor markers and oncogenes (6,7). In one analysis, survival at 5 years in low risk Stage I patients was nearly 90%, but in high risk patients survival was nearly 40% (8). An example of a risk factor is histologic subtype of lung adenocarcinoma, where certain growth patterns such as solid or micropapillary indicate poorer prognosis (9-11). We and others have previously reported that increased SUVmax on pre-treatment fluorodeoxyglucose-PET (FDG-PET) correlate with poorer local control and survival after treatment with SBRT, consistent with surgical series (12). Similar to operable patients, unfavorable subsets of patients have been identified in inoperable patients that are at high risk for nodal and distant failures. These are based on tumor and treatment-related characteristics such as age, functional status, tumor size, histology, proximity to the hilum, and deliverable radiation dose (13).

The opportunity to combine immunotherapy and radiation

There exists a growing body of evidence that T-cell checkpoint inhibitors have robust and enduring activity in some patients with metastatic lung cancer (14-17). Approximately 20% of patients with previously treated lung cancers have objective response to anti-PD-1 or anti-PD-L1 therapies. The responses may be remarkably durable and the treatment associated with good tolerability. Thus far in patients with lung cancers these studies have largely been in patients with metastatic disease, but T-cell checkpoint inhibitors in melanomas have been shown to improve relapse free survival compared to placebo (HR 0.75, P=0.0013) (18). There is a critical unmet need to translate the potential benefits of T-cell checkpoint inhibitors into the early-stage setting for patients with lung cancers. Additionally, as it is only a subset of patients who appear to benefit from anti-PD-1 or anti-PD-L1 therapies, there is also a need to identify effective combination approaches that can augment the benefit of immunotherapy for patients (19,20).

In this context, the opportunity to combine immunotherapy and RT represents a unique approach toward several key challenges in the treatment of patients with lung cancers: (I) can immunotherapy be integrated with RT to improve systemic relapse in patients with early stage lung cancers treated with surgery? (II) can RT in combination with immunotherapy be performed safely and can a synergistic, appropriately sequenced combination be determined?

An analogy to this potential relationship exists in the role of concurrent chemoradiation. In multiple solid tumors, including NSCLC, head and neck disease, and gynecologic malignancies, combined modality therapy with RT and chemotherapy is more effective than either alone, and even more effective compared to sequential therapy. This results in both increased local and systemic control (21). Combined modality therapy has been extensively studied as modulating tumor-host interactions and may improve treatment beyond simply radiosensitization of tumor cells.

Immunotherapy exists in many forms including adoptive T-cell transfer, oncolytic viruses, and cytokine therapy, among other modalities. Currently, immunotherapy using immune checkpoint inhibitors has offered unprecedented rates of response and has since attracted intense attention. Thus the focus of this review will primarily be on immune checkpoint inhibitors and their combination with RT in operable NSCLC.

The activity of immunotherapy in inoperable and metastatic NSCLC

Over 50% of patients with NSCLC will present with metastatic disease and will be treated with chemotherapy with or without local palliative RT. Approximately 18% of patients will present with Stage IIIB unresectable disease and will go on to have chemoradiation with curative intent (22). In these populations, immunotherapy,

despite representing an already heavily treated and frail population with a guarded prognosis, has yielded significant improvements in overall survival (20).

The activity of pembrolizumab was reported in a Phase 1 study that analyzed both efficacy and safety (20). After treatment with pembrolizumab, the objective response rate was 19.4%, and the median duration of overall survival was 12 months. In patients with PD-L1 expression in at least 50% of tumor cells, the objective response rate was 45.2% and median overall survival was not reached. The recently published study of first-line pembrolizumab showed it was superior to chemotherapy in NSCLC patients without sensitizing EGFR mutations or ALK translocations that express the protein PD-L1 in more than 50% of cells (23).

Multiple other studies have shown activity of these and other immune checkpoint inhibitors in NSCLC, which has led to several receiving FDA approval for use in first-line or progressive disease (Hellman ASCO 2016) (24,25). These data also indicate there likely exist tumor-specific characteristics to guide whom may best respond to these therapies.

Immune editing with radiation and improved local control when combined with immune therapy in solid tumors

After treatment, tumor cell transformation drives activation of the host immune response, with modifications in both the innate and adaptive systems. A growing body of evidence suggests that RT can quantitatively augment the immune system by directly upregulating tumor-associated antigens (TAAs), augment MHC class I surface expression in a dose-responsive manner, and increase T-cell tumor-specific CD8+ T cells (26,27). Some tumors downregulate MHC expression to evade immune detection, but upregulation characteristics after RT exposure may prevent this.

Beyond MHC class I surface expression, RT may also stimulate the immune system via activating dendritic cells and increasing antigen cross-presentation. This also increases FAS surface expression, which, in turn, induces programmed cell death. FAS is a cell surface receptor that leads to programmed cell death. FAS upregulation ultimately increases the density of tumor-infiltrating lymphocytes, and upregulates PD-L1 expression (28,29). RT already is known to generate inflammation, increase antigen presentation, and modify the tumor microenvironment. In multiple patient reports, the stimulatory effect of RT inducing an abscopal effect (even in the absence of immunotherapy) has been shown using a variety of RT total doses and fractionation schemes (30).

Multiple preclinical studies show that checkpoint blockade augments the immunostimulatory effects of RT to improve local disease control. Demaria *et al*. showed in breast cancer cell lines that anti-CTLA4 therapy sensitized cells to RT (31). This was additionally demonstrated in an orthotopic glioblastoma model when combining anti-CTLA4 with stereotactic radiosurgery (SRS), a highly potent local therapy. SRS plus checkpoint blockade improved overall survival by 50% as a product of improved local control (32). RT has been shown in melanoma to augment the immune environment leading to the abscopal effect after combination RT and immunotherapy using anti-CTLA4 therapy (33,34). Deng *et al*. first showed that RT upregulated PD-L1, and then showed that anti-PD-L1 therapy enhanced the therapeutic efficacy of ionizing RT. This was primarily accomplished through an enhanced cytotoxic T-cell dependent mechanism. This combined approach also reduced the accumulation of tumor-infiltrating suppressor cells (35). Sharabi *et al*. showed that anti-PD-1 therapy, when combined with stereotactic RT in mouse models of melanoma or breast cancer, increased T-cell infiltration into tumor and enhanced antigen presentation in draining lymph nodes (36).

Combined therapy leading to abscopal and sustained systemic response

While the above mentioned studies suggest that RT and immunotherapy may work synergistically to improve local control, but the clinically unmet need even in patients with localized disease is to improve systemic control given the high propensity for distant progression. Deng *et al*. in the same study as above showed both an abscopal effect and a sustained anti-tumor effect after combined therapy. Using a TUBO breast cancer model, mice receiving both anti-PD-1 therapy and irradiation of a single lesion showed abscopal effect by growth rate reduction of a second unirradiated tumor. After complete tumor eradication, mice were rechallenged with the same tumor and no palpable tumors developed on the dual-treated mice (35). Park *et al*. showed a similar result with melanoma and renal cancer mouse models; however, they further showed that the increased antigenicity was tumor-specific when mice bore both tumors (37).

Postow *et al*. described a patient with metastatic melanoma who was treated with paraspinal SBRT and anti-

CTLA4 therapy and who was later found to have a decrease in non-irradiated splenic and hilar masses (34). Golden *et al.* found a similar effect when a patient with NSCLC was treated with combined therapy. This patient received liver SBRT for a NSCLC metastasis and anti-CTLA4. Not only did the irradiated lesion improve, but there was also significant improvement in nonirradiated disease in the lung, skeleton, and elsewhere in the liver (38).

Multiple other clinical reports show the abscopal effect in patients who have received combined RT and immunotherapy. In one series on the combination of anti-CTLA4 and RT, there was a range of 3–6 months from after treatment until an abscopal effect was reached. A range of 5–47 months was observed from the occurrence of the abscopal effect until further disease progression (39). It is important to note that there was significant heterogeneity in tumor type, site irradiated, and total dose and fractionation of RT. The optimal RT regimen, dose and fractionation to elicit an abscopal effect in combination with immunotherapy remain indeterminate.

Neoadjuvant or adjuvant immunotherapy studies show promise

Several clinical studies have shown activity of various forms of neoadjuvant immunotherapy, including adoptive cell transfer, vaccines, and tumor necrosis factor (TNF) therapies. A study with neoadjuvant chemo-immunotherapy in Stage IIB–IIIB NSCLC patients used cisplatin and gemcitabine, and then randomized patients to concurrent recombinant TNF fused with thymosin-alpha. Seventy-one percent had response to chemo-immunotherapy, versus 50% to neoadjuvant chemotherapy alone (Lazutin ASCO 2015). In an updated analysis, the chemotherapy alone group showed a decrease in NK cells while the chemo-immunotherapy group did not (Zlatnik ASCO 2016).

Kimura *et al.* performed a randomized study of adjuvant chemo-immunotherapy versus immunotherapy alone in patients with IB–IV NSCLC after thoracotomy. Patients who received non-curative resections were included. Chemotherapy was given in platinum-doublets, and immunotherapy consisted of activated killer T-cells and dendritic cells. There was a remarkable difference in 5-year overall survival after the addition of immunotherapy after surgery (81.4% *vs.* 48.3%, HR 0.229, P=0.0013). In addition, there was improvement in recurrence-free survival (40). A meta-analysis of 4 randomized trials consisting of 472 patients showed a significant benefit of adjuvant adoptive immunotherapy with a 39% relative reduction in risk of death. Two of the 4 studies allowed RT as part of treatment (41).

The Radiation Therapy Oncology Group (RTOG) proposed a Phase II study of adjuvant immunotherapy and RT in patients with completely resected Stage II and IIIA NSCLC (RTOG 9909, ClinicalTrials.gov number: NCT00006470). Patients received surgery and within 7 weeks, began two anti-idiotype vaccines (one which mimicked CEA, and the other mimicked the human milk fat globule antigen) and 50.4 Gy in 28 fractions of RT. These vaccines are used to mimic TAAs. Proposed accrual was 54 patients; however, only 22 patients were accrued and the study closed without reporting results.

Current clinical studies in operable patients

There are multiple current clinical trials open exploring neoadjuvant and adjuvant immunotherapy in operable NSCLC, with a significant focus on checkpoint inhibitors given promising results in patients with advanced or metastatic disease. There is significant heterogeneity in the type of immunotherapy utilized, and none currently combine with RT (*Table 1*).

Emerging studies combining radiation and immunotherapy for effectiveness and safety

The prospect of combined modality treatment augmenting curative surgical treatment has significant advantages in NSCLC. Although approaches combining surgery, RT, chemotherapy and immunotherapy are emerging, there is already significant interest in understanding the role of adjuvant immunotherapy after definitive concurrent RT and chemotherapy. The PACIFIC trial aimed to accrue 702 patients with locally advanced NSCLC who received platinum-based chemo-RT, and then were enrolled and randomized to adjuvant durvalumab or observation if they had not progressed after initial therapy (ClinicalTrials.gov number: NCT02125461). This study is closed to accrual and results are expected sometime in 2017. RTOG Foundation 3505 study will enroll patients prior to chemo-RT and randomize 660 patients with stage III NSCLC who will receive chemo-RT followed by adjuvant nivolumab for 1 year, or observation. Both studies will analyze overall survival as their primary endpoint (ClinicalTrials.gov number: NCT02768558).

However, there are unexplored risks to combining chemo-RT, immunotherapy, and surgery. Preoperative RT

Table 1 Active clinical trials using immunotherapy in operable non-small cell lung cancer

Identifier (ClinicalTrials.gov)	Sponsor	Stage	Phase	Immune therapy	Arms	Accrual	Endpoint	Status
NCT02572843	Swiss Group for Clinical Cancer Research	IIIA (N2)	II	Neoadjuvant and adjuvant durvalumab	Cisplatin/docetaxel ± neoadjuvant and adjuvant durvalumab	68	Event-free survival	Open
NCT02818920	Duke University	IB, II, IIIA	II	Neoadjuvant and adjuvant pembrolizumab	Single arm	32	Safety	Open, not yet recruiting
NCT02716038	Columbia University	IB–IIIA	II	Neoadjuvant atezolizumab	Single arm; combined with Nab-paclitaxel and carboplatin	30	Efficacy	Open
NCT02259621	Sidney Kimmel Cancer Center; Memorial Sloan Kettering Cancer Center	I–IIIA	II	Neoadjuvant nivolumab	Single arm	20	Safety	Open
NCT02595944	NCI-EA5142	IB–IIIA	III	Adjuvant nivolumab	Postoperative platinum-based chemotherapy as per standard of care, ± PORT ± nivolumab	718	DFS/OS	Open
NCT02273375	Canadian Cancer Trials Group	IB–IIIA	III	Adjuvant durvalumab	Postoperative platinum-based chemotherapy as per standard of care (no RT) ± durvalumab	1,100	Disease-free survival	Open

NCI, National Cancer Institute; RT, radiation therapy; DFS, disease-free survival; OS, overall survival; PORT, postoperative radiation therapy.

may lead to lung fibrosis or lung edema, resulting in difficult surgery or concerns with wound healing (42). Postoperative RT, particularly in those who have had significant lung volume removed may have compromised lung function from pneumonitis or long-term pulmonary fibrosis.

Some potential RT toxicities may be augmented by immunotherapy, or vice versa. Grade 3 or higher pneumonitis may be seen in 2% of patients after treatment with pembrolizumab, and it is unknown the magnitude of synergy with RT and immune checkpoint modulators on pneumonitis (20). Published data on RT and immunotherapy in other disease sites suggests no significant increase in the risk of toxicity. This includes no significant increased toxicity of immunotherapy when combined with brain SRS or with pelvic RT (43-45).

Our group has shown that combining thoracic RT and immunotherapy is generally safe and yields acceptable toxicities within the range of treatment with thoracic RT alone. The most often encountered toxicities included fatigue, infection, dermatitis, and rash. Pneumonitis, primarily Grade 1 and 2, occurs in approximately 7% of patients. There were no differences in toxicity when comparing patients who received immunotherapy concurrently or sequential with RT (46).

Challenges to this approach

There is tremendous potential benefit to combining RT and immunotherapy with surgery. Given that the bulk of patients fail distantly, improving systemic relapse rates is of critical importance. However, there are significant emerging challenges in this approach.

First, we must determine which patients are most likely to benefit from this combined modality treatment by (I) identifying patients likely to fail and (II) identifying patients who will respond to RT and immunotherapy. Previous data shows that patients with high pre-SBRT SUV_{max} or adenocarcinoma subtype (e.g., micropapillary or solid) may be the most likely to fail local therapy, and other clinico-pathologic markers such as mutation status may enter into consideration (11,12). In addition, not all patients may respond to RT or immunotherapy. There remains discord in identifying patients who may or may not benefit from immunotherapy and what are the best methods to determine this metric (e.g., CD8 T-cells, total lymphocytes, PD-L1 expression, IL-6 plasma levels, etc.) (47).

For example, studies showing patient's with tumors expressing >50% PD-L1 may be the best responders to certain immunotherapies (20).

After patients are identified, it is unclear what treatment schema to use. There are multiple forms of immunotherapy including multiple checkpoint inhibitors with various targets, vaccines, and adoptive T-cell transfer, among others. There is no consensus in what RT total dose and fractionation to use. Finally, the timing of each of these treatments also lacks clarity. It is unclear whether how best to order therapy whether sequential or concurrent RT and immunotherapy, or if best to use these therapies in the adjuvant versus neoadjuvant setting in relation to surgery. The neoadjuvant setting allows determination of initial tumor response, and possible guidance on post-operative systemic therapy (48).

Once patients are treated with this combined modality approach, it is unclear exactly how to measure treatment response. The most obvious is clinical and imaging evidence of progression-free survival after surgery, RT, and immunotherapy. Even with routinely used imaging modalities responses to immunotherapy can present in unusual fashion such as delayed responses, pseudoprogression etc. Therefore immune-related response criteria were developed and are analyzed in many prospective studies to further evaluate the natural presentation of these immunotherapy responses (49). However, other biomarkers of treatment response should play also be included. These markers may perhaps include measures of immune response and measures of tumor response (e.g., circulating tumor DNA).

There remain a number of concerns in regards to the above with efficacy, timing, type of immunotherapy, dose and location of RT, and measuring response. However, there remains great promise in this approach combining the immune-stimulatory effects of both RT and immunotherapy to decrease systemic relapse rates in patients with otherwise curable disease.

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Footnote

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Lung cancer biomarkers, targeted therapies and clinical assays

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Abstract: Until recently, the majority of genomic cancer research has been in discovery and validation; however, as our knowledge of tumor molecular profiling improves, the idea of genomic application in the clinic becomes increasingly tangible, paralleled with the drug development of newer targeted therapies. A number of profiling methodologies exist to identify biomarkers found within the patient (germ-line DNA) and tumor (somatic DNA). Subsequently, commercially available clinical assays to test for both germ-line and somatic alterations that are prognostic and/or predictive of disease outcome, toxicity or treatment response have significantly increased. This review aims to summarize clinically relevant cancer biomarkers that serve as targets for therapy and their potential relationship to lung cancer. In order to realize the full potential of genomic cancer medicine, it is imperative that clinicians understand these intricate molecular pathways, the therapeutic implication of mutations within these pathways, and the availability of clinical assays to identify such biomarkers.

Keywords: Assay; biomarker; lung cancer; mutation; pharmacogenetic

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Introduction

Given the large heterogeneity in clinical response observed across cancer patients and the narrow therapeutic indices of anticancer drugs, novel methods for individualizing cancer therapy are critical to improve patient outcomes. Our understanding of cancer at the molecular level has resulted in a shift from characterizing tumors solely by anatomical location to consideration of their molecular profile (1). Until recently, the majority of genomic cancer research has been in discovery and validation; however, as our knowledge of tumor molecular profiling improves, genomic cancer medicine in the clinic becomes increasingly tangible (2). As the number of commercially-available clinical assays to test for tumor biomarkers increases, it is critical that clinicians understand the therapeutic implications of mutations occurring within these molecular pathways. This review aims to summarize clinically relevant cancer biomarkers, their potential relationship to lung cancer and the clinical assays available in practice to test for such biomarkers (*Table 1*).

Biomarkers review

Biomarker classification

DNA analysis for pharmacogenetic purposes can be performed with either somatic or germ-line DNA. Somatic mutations are found within the tumor, requiring a tumor biopsy for identification, and are particularly useful in evaluating pharmacodynamic effects of a drug, such as tumor response. Germ-line, or inherited, variations are identified by a peripheral blood sample and help to predict the pharmacokinetic behavior of a drug, and ultimately drug response (3). Cancer biomarkers can be broadly categorized into two classifications: prognostic and predictive. A prognostic biomarker is mainly associated with disease outcome in the absence of treatment (i.e., Oncotype Dx, Mammaprint), while a predictive biomarker is valuable in assessing drug response [i.e., anaplastic lymphoma kinase (*ALK*), epidermal growth factor receptor (*EGFR*), Kirsten rat sarcoma viral oncogene homolog (*KRAS*)] (4). Biomarkers may also be classified as both prognostic and predictive [i.e., human epidermal growth factor receptor-2

Table 1 Select cancer biomarkers, targeted therapies, and clinical assay availability

Biomarker	Targeted therapy	Tumor	Clinical assay(s) available	Molecular profiling methodology
ALK/ROS1	Crizotinib, ceritinib	Lung	Vysis ALK Break Apart FISH probe kit[a]	FISH
BRAF (V600E)	Vemurafenib, dabrafenib, trametinib	Lung, melanoma	Cobas 4800 BRAF V600E Mutation Test[a]; THxID BRAF test[a]	Real time PCR
C-KIT	Imatinib mesylate	Lung, GIST	C-KIT pharmDx[a]	IHC
EGFR	Erlotinib, afatinib	Lung, colorectal	EGFR pharmDx[a], Therascreen EGFR RGQ PCR kit[a]; Cobas EGFR Mutation Test[a]	IHC, Sanger Sequencing, PCR
HER2 (ERBB2)	Trastuzumab, lapatinib, pertuzumab, ado-trastuzumab-emtansine, dacomitinib	Lung, breast	HercepTest[a], Pathway[a], Insite[a], PathVysion[a], SPOT-Light[a], HER2 CISH[a]	IHC, FISH, CISH
JAK2	Ruxolitinib	Lung, myelofibrosis and other myeloproliferative disorders	JAK2 V617F Mutation Detection Assay, HTScan JAK2 Kinase Assay Kit	Real time PCR, Kinase activity assay
PD-1	Pembrolizumab, nivolumab	Lung, melanoma	In development	N/A
KRAS	Cetuximab, panitumumab	Lung, colorectal	Therascreen KRAS RGQ PCR Kit[a], DxS KRAS Mutation Test Kit, Genzyme's KRAS Mutation Analysis	Real time PCR

[a], assays that are FDA approved, PMA or 510(k) status. IHC, immunohistochemistry; HER2, human epidermal growth factor receptor-2; CISH, chromogenic in situ hybridization; FISH, fluorescence in situ hybridization; PCR, polymerase chain reaction; EGFR, epithelial growth factor receptor; GIST, gastrointestinal stromal tumor; ALK, anaplastic lymphoma kinase; JAK2, janus kinase 2; PD-1, programmed cell death 1; ROS1, ROS proto-oncogene 1, receptor tyrosine kinase.

(*HER2*), B-Raf proto-oncogene, serine/threonine kinase (*BRAF*)]. Pharmacodynamic biomarkers, a subset of predictive biomarkers, are useful in measuring the treatment effects of a drug on the tumor or on the host and can be used to guide dose selection. Examples include thiopurine-S-methyltransferase (*TPMT*) to guide 6-mercaptopurine dosing and uridine-diphosphate glucuronosyl transferase 1A1 (*UGT1A1*) to guide irinotecan dosing (5).

Lung cancer is the leading cause of cancer-related mortality worldwide. Molecularly targeted therapies have dramatically improved the ability to extend survival in patients with lung cancers positive for *EGFR* mutations and/or *ALK* translocations. Researchers in The Cancer Genome Atlas Network molecularly profiled 230 resected lung adenocarcinomas using messenger RNA, microRNA and DNA sequencing integrated with copy number, methylation and proteomic analyses. Results demonstrated high rates of mutations at a mean of 9 per megabase, while 18 genes were statistically significantly mutated including *RIT1*, *EGFR*, *NF1*, *MET*, *ERBB2*, *RBM10*, and others within the mitogen-activated protein kinase (*MAPK*) and phosphatidylinositol-3-kinase (*PI3K*) pathways (6). Although several genes identified are not currently druggable and their prognostic significance has yet to be elucidated, understanding these molecular pathways and their predictive potential are critical to advancing personalized lung cancer therapy. The remaining article will focus on cancer biomarkers for which targeted therapies are available, their influence on lung cancer therapy, and, lastly, potential new targets for drugs in the pipeline.

Cancer biomarkers and lung cancer

Anaplastic lymphoma kinase (ALK)

Activating translocations of *ALK* resulting in the abnormal fusion gene, *EML4-ALK*, occurs in approximately 2-7% of all non-small cell lung cancer (NSCLC) cases, and encodes

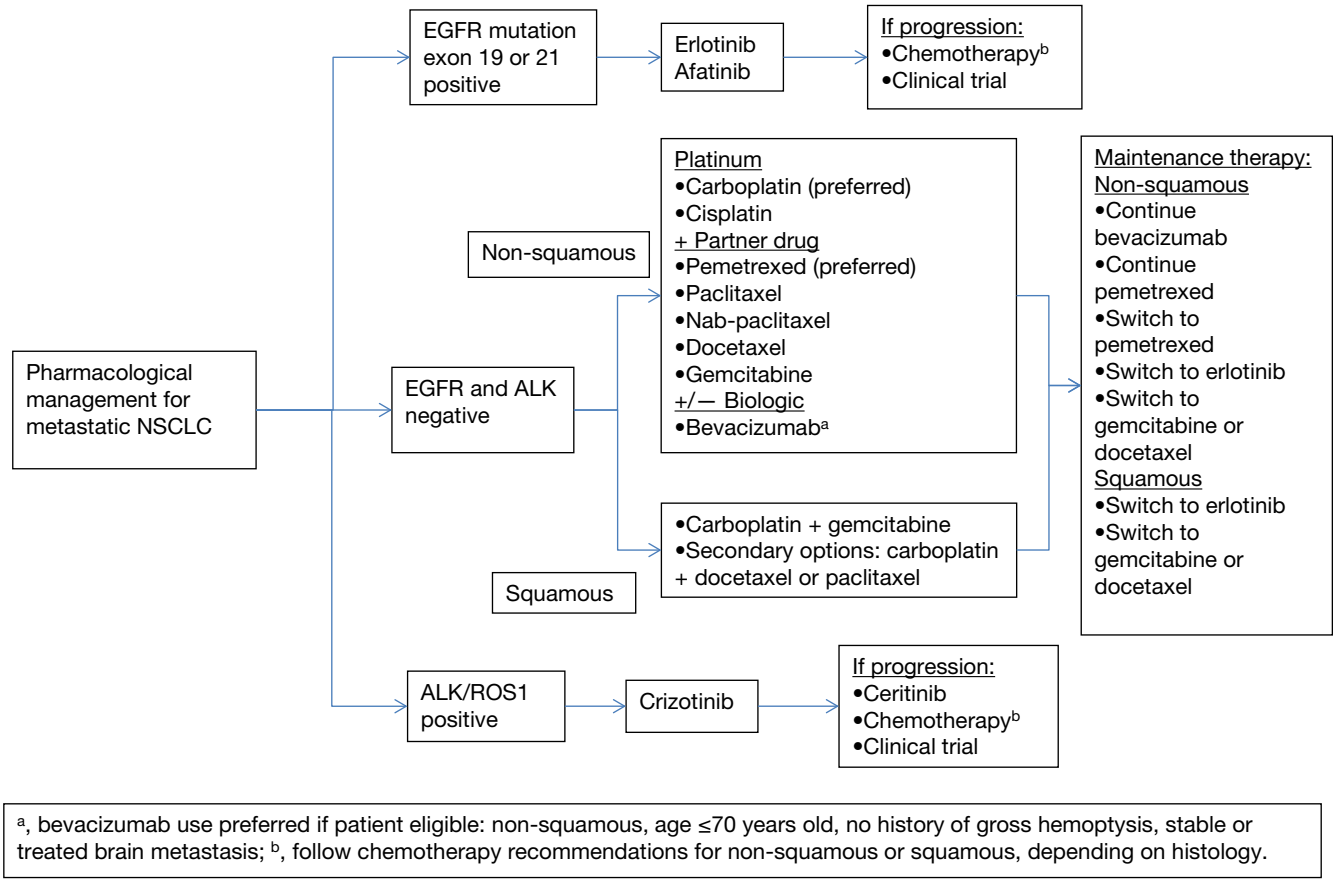

Figure 1 Example of a biomarker-driven treatment pathway for NSCLC, whereby mutations in EGFR or ALK drive targeted therapy selection, while patients with tumors negative for these biomarkers have therapy guided by histology and other clinical factors. NSCLC, non-small cell lung cancer; ALK, anaplastic lymphoma kinase; EGFR, epidermal growth factor receptor; ROS1, ROS proto-oncogene 1, receptor tyrosine kinase.

a cytoplasmic chimeric protein with constitutive kinase activity allowing activation of the *RAS-MEK-ERK*, janus kinase 3 (*JAK3)-STAT3*, and *PI3K-AKT* pathways (7). Similar to *EGFR* mutations, *ALK* rearrangements in NSCLC are associated with clinical and histopathologic features, such as adenocarcinoma histology and nonsmoking history. In contrast to *EGFR* mutations, patients with *ALK* rearrangements tend to be significantly younger and male, with no significant differences in frequency between Asian and Western populations (8). Treatment with crizotinib, a tyrosine kinase inhibitor (TKI) that competitively binds to *ALK*, demonstrated an initial overall response rate (ORR) of 60.8% in *ALK*-positive NSCLC patients treated in a phase I clinical trial, advancing the molecule into an accelerated FDA approval process (7). Results from the randomized phase III trial comparing crizotinib versus docetaxel/pemetrexed in *ALK*-positive NSCLC unequivocally demonstrated that crizotinib results in improved ORR (65% *vs.* 20%; P<0.05) and median progression-free survival (PFS) (7.7 *vs.* 3.0 months; P<0.05) (9). *Figure 1* illustrates a targeted approach to therapy selection in NSCLC based on clinically relevant biomarkers, including *ALK* and *EGFR* (discussed later in the article).

Although the majority of patients with *ALK*-positive NSCLC derive substantial benefit from crizotinib, this benefit is relatively short-lived secondary to acquired resistance. Possible mechanisms of resistance may include novel *EGFR*, *KIT*, *MET*, ROS proto-oncogene 1, receptor tyrosine kinase (*ROS1*) or secondary *ALK* mutations not previously identified (10). Ceritinib, a second generation *ALK* inhibitor with greater potency compared to crizotinib, received accelerated FDA approval for the treatment of metastatic *ALK*-positive NSCLC in patients who were previously treated with crizotinib. A phase I study

demonstrated ORRs of 58% and 56% in crizotinib naïve and resistant cases, respectively (11). As evident by crizotinib and ceritinib, the drug development paradigm for highly targeted therapies is changing, allowing earlier, accelerated approval of exceedingly effective therapies, years before phase III randomized studies are completed. Additionally, companion diagnostic test approval will become increasingly common with targeted therapy approval, particularly for newly identified biomarkers [i.e., Vysis *ALK* Break Apart fluorescence in-situ hybridization (FISH) Probe Kit to detect *ALK* rearrangements].

Lastly, evidence suggests that patients with *ALK*-positive NSCLC have improved survival after radiotherapy for brain metastases compared with *EGFR*, *KRAS* or wild-type tumors. The median overall survival (OS) was 13.6, 26.3, 5.7 and 5.5 months in patients with *EGFR*, *ALK*, *KRAS* or wild-type tumors. Subsequent receipt of targeted therapy was also associated with additional improvement in OS (12).

BRAF gene

BRAF mutations have been identified in a wide range of cancers including 50% of malignant melanomas, 45% of papillary thyroid cancers, 10% of colorectal cancers, and 3% of lung cancers (13). Mutations in *BRAF* result in constitutive activation of downstream signaling through the *MAPK* pathway (14). Approximately 50-90% (depending on anatomical location) of these mutations result in the substitution of glutamic acid for valine at codon 600 (*V600E*) (15). In contrast to lung cancer patients with *EGFR* mutations and *ALK* rearrangements who are mostly never smokers, patients with *BRAF* mutations tend to be current or former smokers.

Vemurafenib, a potent and selective *BRAF V600E* inhibitor, and its companion diagnostic test (Cobas 4800 BRAF V600 Mutation Test) received accelerated FDA approval upon demonstrating significant improvements in OS and PFS compared to dacarbazine in metastatic melanoma patients harboring the *BRAF V600E* mutation [hazard ratio (HR) =0.37 for OS, HR =0.26 for PFS; P<0.001 for both] (14). Patients with *BRAF*-mutated colorectal tumors tend to have significantly shorter PFS and OS compared to wild-type patients, and also have the potential to impair the effects of EGFR-inhibitor therapy in *KRAS* wild-type patients (15). However, no benefits with vemurafenib were noted in colorectal cancer, indicating the significance of tumor origin and microenvironment (16). The data for *BRAF* inhibition in lung cancer is scarce, although case reports have demonstrated clinical activity with vemurafenib (complete response after 6 weeks of therapy in a patient with refractory stage IV NSCLC) (17). Another case report demonstrated clinical activity in a metastatic NSCLC patient with brain metastases, with regression of both visceral and intracranial disease (18). Interim results of a phase II study of dabrafenib in *BRAF V600E*-positive NSCLC patients who failed at least one line of chemotherapy showed early antitumor activity with an ORR of 54% (19).

A number of mechanisms have been elucidated for BRAF resistance, including the paradoxical activation of the MAPK pathway through *RAS* mutations (20). Studies have demonstrated significantly improved OS and PFS in metastatic melanoma patients receiving a concomitant mitogen-activated protein/extracellular signal-regulated kinase (MEK) inhibitor, trametinib, in combination with a selective BRAF inhibitor, dabrafenib (21). Both drugs received FDA approvals in 2013 for the treatment of patients with unresectable or metastatic melanoma with *BRAF V600E* or *V600K* mutation who have not already received a *BRAF* inhibitor. Similar mechanisms of resistance may be translated to lung cancer. A randomized phase II trial of docetaxel with and without the *MEK* inhibitor selumetinib revealed that the combination resulted in superior OS, and a statistically significant improvement in PFS and objective response rate (22). Based on promising preclinical data (23), combination of targeted therapies, such as dabrafenib plus trametinib, may ultimately prove useful in treating *BRAF*-positive NSCLC and should be explored further.

C-KIT gene

The *C-KIT* proto-oncogene encodes a receptor tyrosine kinase, which binds to stem cell factor ligand. This interaction allows for the development of melanocytes, erythrocytes, germ cells, and mast cells, ultimately resulting in dimerization, autophosporylation, and signal transduction (24). While gain-of-function *C-KIT* mutations are found in approximately 85% of gastrointestinal stromal tumors (GIST) and are predictive of response to imatinib therapy (25), research suggests approximately 40% of small-cell lung cancers (SCLC) overexpress *C-KIT* (26). However, expression of *C-KIT* in SCLC failed to demonstrate a significant impact as a predictive biomarker of survival, possibly due to tumor microenvironment, resulting in

futility of target inhibition in this setting (26). Alternatively, evidence suggests *C-KIT* mutations may be a prognostic factor for worse survival (27). Current literature on *C-KIT* inhibition in SCLC is limited and continued researches on its prognostic and predictive value are necessary.

Epidermal growth factor receptor (EGFR)

Activating *EGFR* mutations result in constitutive signaling via the PI3K-AKT and RAS-MEK-ERK pathways (28). Deletions in exon 19 and a missense mutation at exon 21, resulting in an arginine to leucine substitution (L858R), account for 90% of all *EGFR* mutations. Approximately 15-20% of NSCLCs harbor mutated *EGFR*, resulting in significantly improved PFS and OS when treated with small molecule TKIs targeting the *EGFR* domain (erlotinib, gefitinib, afatinib) compared to traditional platinum-based chemotherapy (29). Zhou *et al.* prospectively tested NSCLC patients for mutated *EGFR* and evaluated first-line erlotinib versus chemotherapy (30). Median PFS was significantly longer in erlotinib-treated patients compared to those receiving chemotherapy (13.1 *vs.* 4.6 months, HR 0.16, 95% CI, 0.10-0.26; P<0.0001). The ORR was 83% and 36% for erlotinib and chemotherapy-treated patients, respectively (30). Subgroup analyses from clinical trials revealed that patients with certain clinical and histologic characteristics (female, patients of East Asian descent, non-smokers, and those with adenocarcinomas) are more likely to harbor *EGFR* mutations (31,32).

Currently, screening for *EGFR* mutations is used to select stage IV NSCLC patients that should receive erlotinib in the first-line setting. In 2013, the FDA approved a companion diagnostic test for erlotinib (Cobas *EGFR* Mutation Test) and authorized expanded approval for first-line use in patients with metastatic NSCLC that tests positive for the *EGFR* activating mutation (33). Also in 2013, a second generation *EGFR* inhibitor, afatinib, received FDA approval for the first-line treatment of patients with metastatic NSCLC whose tumors have *EGFR* mutations. Afatinib's irreversible binding mechanism of action allows for enhanced activity in resistant tumors that have progressed after initial *EGFR* inhibitor therapy (34). In a phase III trial, 1,269 NSCLC patients with *EGFR* mutations were randomized to receive afatinib or standard chemotherapy (cisplatin and pemetrexed). The median PFS was 11.1 and 6.9 months in the afatinib and chemotherapy arms, respectively (35).

Two primary mechanisms of resistance to *EGFR* inhibitors include a secondary point mutation in *EGFR* (*T790M*) that blocks the capacity for erlotinib to inhibit the receptor, and the amplification of *MET*, which activates similar downstream signaling pathways (36). Drugs targeting *EGFR T790M* mutations and *MET* amplifications are currently under development.

Human epidermal growth factor receptor-2 (HER2)

HER2 is one of the molecular hallmarks of breast cancer and has resulted in the development of several successful targeted therapies. *HER2* or *ERBB2*, is a member of the ERBB receptor tyrosine kinase family, which includes three additional members: *EGFR* (*HER1/ERBB1*), *HER3* (*ERBB3*) and *HER4* (*ERBB4*). The binding of ligands to the extracellular domain of these receptors results in dimerization, activating a catalytic cascade of events involved in cellular proliferation, differentiation and migration. *HER2* status represents both a prognostic and predictive biomarker as overexpression is associated with higher breast cancer recurrence and mortality rates without consideration of pharmacological therapy; however, *HER2* overexpression also predicts response to anti-*HER2* targeted therapies, which has resulted in drastic improvements in median survival (37). Overexpression of *HER2* may be diagnosed using immunohistochemistry (IHC) analysis (for protein expression) or FISH (for gene expression).

Trastuzumab, the first monoclonal antibody targeting the extracellular domain of *HER2*, was approved in 1998 as first-line treatment in combination with paclitaxel for *HER2*-positive advanced and metastatic breast cancer (38). Lapatinib, a small molecule TKI targeting the intracellular domain of *HER2*, resulted in extended survival in metastatic *HER2* positive breast cancer in combination with capecitabine compared to capecitabine alone (39). Pertuzumab, an anti-*HER2* humanized monoclonal antibody that inhibits receptor dimerization, prolonged PFS in metastatic breast cancer patients when combined with trastuzumab and docetaxel compared to trastuzumab and docetaxel alone (40). Trastuzumab emtansine (T-DM1), an antibody-drug conjugate combining the targeted strategy of trastuzumab with the cytotoxic properties of emtansine, prolonged PFS and OS in patients with *HER2* positive, advanced BC previously treated with trastuzumab and a taxane (41).

Although HER2 overexpression and amplification has been described in 6-35% and in 10-20%, respectively, of NSCLC patients, the first clinical trials including patients

treated with trastuzumab and gemcitabine-cisplatin or docetaxel, failed to demonstrate an OS benefit in *HER2*-positive patients (42,43). *HER2* mutations have been reported to exist in approximately 1-4% of NSCLC and are more common in Asians, non-smokers, women and those with adenocarcinomas (44). Considering that *HER2*-positive NSCLC may benefit from *HER2* inhibition or dual *EGFR/HER2* inhibitions, TKIs simultaneously targeting *EGFR/HER2* have been investigated. Case reports of afatinib in patients with *HER2*-positive NSCLC have suggested promising outcomes. Of five patients harboring *HER2* mutations, three observed objective responses (45). However, studies with neratinib, an irreversible pan ERBB inhibitor, suggested no benefit in response in *HER2*-positive NSCLC (44). Lastly, dacomitinib, another irreversible ERBB inhibitor, has demonstrated a 14% partial response rate in *HER2*-positive NSCLC (46). Continued research in larger patient populations will provide a better understanding of the clinical utility of *HER2* (or pan-*ERBB*) inhibition in *HER2* positive NSCLC.

Janus kinase 2 (JAK2)

JAKs are non-receptor TKs that mediate the transmission of cytokine and growth-factor-induced intracellular signals. The mutation is a single nucleotide change, resulting in a valine to phenylalanine substitution at codon 617, and occurs in approximately 55% of patients suffering from myeloproliferative disorders (47). The transcription of numerous pro-proliferative and anti-apoptotic genes are up-regulated upon activation of the JAK-STAT pathway. Ruxolitinib is the first *JAK* inhibitor approved by the FDA for treatment of patients with myelofibrosis or myeloproliferative disorders. In the COMFORT-II trial, the proportion of patients achieving at least a 35% reduction in spleen volume at week 48, was 28.5% for ruxolitinib and 0% for best available therapy (P<0.0001) (48).

Although *JAK* mutations in NSCLC are rare, data suggests that the activation of *JAK2* partially accounts for acquired erlotinib resistance. The combination of *JAK2* inhibition with erlotinib in erlotinib-resistant lung cancer cell lines demonstrated restored sensitivity to erlotinib and reduction in tumor size in a murine xenograft model (49). Another study demonstrated a commonly mutated pathway in solid tumors, STAT3, is activated by *JAK2* independent of other key oncogenic drivers in NSCLC; however, treatment with ruxolitinib in STAT3-activated NSCLC cell lines did not result in growth inhibition (50). Clinical trials are currently underway to investigate the influence of *JAK2* inhibition with ruxolitinib in NSCLC patients receiving chemotherapy or erlotinib (ClinicalTrials.gov NCT02119650 and NCT02155465, respectively).

KRAS gene

Mutations of the *KRAS* oncogene have emerged as a powerful negative predictive biomarker to identify patients with metastatic colorectal cancer who do not benefit from *EGFR*-inhibitor therapies, such as panitumumab and cetuximab. Roughly 40% of colorectal tumors harbor a *KRAS* mutation (51). *KRAS* functions as a mediator between the extracellular ligand binding and intracellular signal transduction from the *EGFR* and nucleus (52). The autophosphorylation of the intracellular TK domains at codons 12 and 13 of exon 2 confers constitutive activity of downstream signaling pathways, including RAS-RAF-MAPK and PI3K-AKT pathways (51). Significant improvements in PFS were seen in *KRAS* wild-type colorectal cancer patients receiving *EGFR*-inhibitor therapy in combination with FOLFOX or FOLFIRI, while PFS was reduced in patients harboring *KRAS* mutations (53,54).

A meta-analysis of *KRAS* mutations in NSCLC described a frequency of 26% in tumors of current/former smokers, and 6% in tumors of never smokers (55). *KRAS* mutations have been identified as a predictor of resistance to EGFR-TKIs in NSCLC (56). While patients with *KRAS* mutated tumors experienced a suboptimal response to EGFR-TKIs, *KRAS* mutation status did not appear to affect OS (57). *KRAS* mutations are typically mutually exclusive of *EGFR* mutations and *ALK* translocations. While it has traditionally been extremely difficult to develop drugs to specifically target *KRAS* mutations, recent advances have been made to identify downstream pathways and co-mutations that indirectly affect *KRAS*, such as *STK11* and *TP53*. Early research suggests that a MEK inhibitor plus docetaxel can effectively target these co-mutations. In a preclinical study, *KRAS* mutated mice (also mutated for *STK11* and *TP53*) were treated with docetaxel alone or with an investigational MEK inhibitor, selumetinib (58). Concomitant loss of either *TP53* or *LKB1* markedly impaired the response of *KRAS*-mutant cancers to docetaxel monotherapy. The addition of selumetinib provided substantial benefit for mice with lung cancer caused by *KRAS* and *KRAS*-plus-*TP53* mutations, though mice with co-mutations in *KRAS* and *LKB1* were resistant to the combination. A phase II randomized trial of

selumetinib plus docetaxel in *KRAS*-mutant NSCLC patients demonstrated a PFS of 5.3 months with the combination versus 2.1 months with docetaxel alone (P<0.05). Response rates were 37% and 0%, and median OS times were 9.4 and 5.3 months, respectively (22). Another oral MEK1/MEK2 inhibitor, trametinib, demonstrated efficacy in combination with docetaxel in *KRAS*-mutant and wild-type NSCLC (59). Confirmatory clinical trials are ongoing to validate the use of these agents in *KRAS*-mutant NSCLC.

Programmed cell death 1 (PD-1), programmed death-ligand 1 (PD-L1), PD-L2

Cancer immunotherapy rests on the premise that tumors can be recognized as foreign rather than self and can be effectively attacked by an activated immune system. However, during tumor progression, acquisition of traits that allow cancer cells to evade immune surveillance may occur by exploiting checkpoints that control the regulatory immune response (60). PD-1 receptor is an inhibitory receptor that is expressed by T cells with its ligand (PD-L1) found in the tumor microenvironment and a second ligand, PD-L2, expressed by antigen presenting cells (61). PD-L1 and PD-L2 have been shown to down-regulate T-cell activation upon binding to PD-1, especially in cancer, thus interrupting immune response (62).

Pembrolizumab is a highly selective, humanized monoclonal IgG4-kappa isotype antibody that acts against PD-1and blocks the negative immune regulatory signaling of the PD-1 receptor (61,63). Pembrolizumab has been investigated in a number of tumor types, mostly melanoma, but also NSCLC, sarcoma, carcinoid, colorectal, prostate, breast, ovarian, gastric, pancreatic and renal cell cancer (61,63-65). Grade 3 or 4 adverse events have included elevated aminotransferase, renal failure, diarrhea, hypothyroidism, fatigue, abdominal pain, decreased appetite, rash, pruritis (61). Pembrolizumab received accelerated FDA approval in September 2014 for the treatment of melanoma in patients with unresectable or metastatic disease who have disease progression following treatment with ipilimumab and, if BRAF V600 mutation positive, a BRAF inhibitor. In a phase I study of 450 NSCLC patients who had received prior chemotherapy, 159 patients had tumors with strong PD-L1 expression and received pembrolizumab 10 mg/kg IV every 3 weeks. The response rate was 23% with duration of response of 31 weeks. However, in 35 patients with tumors that were PD-L1 negative, the response rate was 9% (66). Further work is ongoing to determine the predictive nature of PD-L1 expression.

Priority review and breakthrough status was granted for nivolumab (an anti-PD-1 antibody) after investigators demonstrated significantly better response and survival outcomes with nivolumab compared to investigator's chemotherapy in the second line treatment of patients with advanced melanoma. Subsequently, the FDA expanded the approved use to treat metastatic squamous cell NSCLC in patients who have progressed on or after platinum-based chemotherapy. In a phase I trial with expansion cohorts of 129 NSCLC patients receiving nivolumab (1 mg/kg, 3 mg/kg, or 10 mg/kg IV every 2 weeks), the ORR was 17.1% and appeared similar between squamous and non-squamous histologies. A difference in ORR between dose levels was observed: 3% for 1 mg/kg, 24.3% for 3 mg/kg and 20.3% for 10 mg/kg. The median PFS and OS were 2.3 and 9.6 months, respectively. One year after starting therapy, 42% of patients were still alive and durable responses were common with a median duration of response of 74 months (65). CheckMate-017, a phase III randomized study comparing second-line docetaxel to nivolumab (3 mg/kg) in patients with squamous cell NSCLC, was stopped early as the Data Monitoring Committee deemed that the trial had met its primary endpoint, demonstrating superior OS in patients treated with nivolumab (67). Currently, no validated marker exists to identify patients most likely to respond to anti-PD-1 therapy; however, continued investigations into the predictive value of PD-1 and PD-L1 expression is ongoing.

Investigational cancer biomarkers and lung cancer

c-MET

Signaling through the c-MET/human growth factor (HGF) pathway has been shown to trigger a variety of cellular responses, including growth, motility, metastasis, angiogenesis and tissue regeneration (68). High levels of HGF have been associated with more aggressive biology and a worse prognosis in NSCLC and SCLC. *c-MET* is normally expressed by epithelial cells and has been found to be overexpressed and amplified in a variety of human tumor tissues. Furthermore, the *c-MET* pathway is one of the key players in the development of acquired resistance to the vascular endothelial growth factor (VEGF) pathway inhibitors (68). Tumor microarray expression analysis demonstrated 72% *c-MET* expression in human lung cancer tissue and 40% *c-MET* receptor over-expression. Acquired *c-MET* amplification has also been linked to approximately

22% of non-*T790M* mediated secondary gefitinib resistance in NSCLC patients (69).

A selective *c-MET* inhibitor, tivantinib, has been studied in three phase I trials, either alone or in combination with erlotinib (68). The combination regimen was further studied in a phase II randomized study, which demonstrated a median PFS of 3.8 months in the combination arm versus 2.3 months in the erlotinib arm (HR 0.81, P=0.24), with no significant difference in ORR or OS (70). However, a trend towards greater benefit with the addition of tivantinib was evident in patients with *c-MET* positive tumors. Continued work is ongoing to further assess this agent in NSCLC. Non-selective *c-MET* inhibitors include crizotinib and cabozantinib. Crizotinib was initially synthesized as a *c-MET* inhibitor; however, after observing dramatic response in *ALK*-positive NSCLC, this drug essentially became recognized as an *ALK* inhibitor (68). Early, phase I data suggest adding cabozantinib to erlotinib is safe and effective, and is currently being explored in phase II trials. Lastly, *c-MET* targeted monoclonal antibodies are being studied in this setting, including onartuzumab (MetMab) (68). Phase II data suggests prolonged PFS (3.0 *vs.* 1.5 months; HR 0.47; P=0.01) and OS (12.6 *vs.* 4.6 months; HR 0.37; P=0.002) in patients with *c-MET* positive NSCLC receiving MetMab plus erlotinib versus erlotinib alone (71). As such, a phase III trial is ongoing to validate these findings.

Fibroblast growth factor receptor (FGFR)

The *FGFR* tyrosine kinase family is comprised of four kinases, *FGFR1*, *2*, *3*, and *4*, that play a critical role in cell survival and tumor growth. Genetic alterations of *FGFRs* can lead to deregulated activation in various cancers, including breast, colorectal, bladder, in addition to lung cancer and others. A pan-*FGFR* TKI has been shown to block tumor proliferation in a subset of NSCLC cell lines with activated *FGFR* signaling but has no effect on cells that do not activate the pathway (72). A study demonstrated that *FGFR1* is amplified in 21% of lung squamous cell carcinomas and 3.4% of lung adenocarcinomas (73), suggesting *FGFR1* may be a potential target in mutation-positive lung cancers. In a phase I study, a selective pan-*FGFR* inhibitor demonstrated safety in patients with *FGFR*-positive squamous cell carcinoma of the lung. Early analysis demonstrated partial responses; however, robust efficacy data is not yet published (74). Another phase I trial is ongoing to assess *FGFR* inhibition in patients with a variety of solid tumors, including *FGFR* positive lung cancer (NCT01962532).

PIK3CA

The PI3K pathway is related to tumor growth in a variety of human cancers. PI3K-dependent activity is frequently elevated due to mutations of *PIK3CA*, the gene encoding PI3K, in addition to the loss of phosphatase and tensin homolog (PTEN) protein, a tumor suppressor with a critical role in regulating the PI3K pathway. *PI3KCA* activation initiates events leading to phosphorylation of Akt, which affects additional downstream signaling proteins involved in cell growth, metabolism, proliferation, survival, motility, and invasion (75). In one study, *PIK3CA* mutations in NSCLC were found in 3.9% of squamous cell carcinoma and 2.7% of adenocarcinoma. Furthermore, among *PIK3CA* mutant cases, about 50% of tumors harbored concurrent *EGFR* mutations and 10% had *KRAS* mutations. *PIK3CA* mutation was significantly associated with high expression of PI3K, p-Akt and mTOR, but not correlated with *PIK3CA* amplification. Patients with single *PIK3CA* mutation had shorter OS than those with *PIK3CA-EGFR/KRAS* co-mutation or wild-type *PIK3CA* (P=0.004). A significantly worse survival was also found in patients with *PIK3CA* mutations than those without *PIK3CA* mutations in the *EGFR/KRAS* wild-type subgroup (P=0.043), suggesting that *PIK3CA* mutations confer a worse prognosis (76).

A preclinical study demonstrated that targeted inhibition of *PIK3CA* in SCLC models harboring *PI3KCA* mutations resulted in cell apoptosis, inhibition of cell viability, transformation, and xenograft tumor growth, suggesting a potential role for *PI3KCA* inhibitors in mutated SCLC (77). Ongoing or recently completed trials in lung cancer include single-agent PI3K inhibitors (NCT01501604), as well as combinations with chemotherapy (NCT00974584, NCT00756847) (78).

Conclusions

The implementation of genomic cancer medicine relies on the foundation that genetic aberrations exist in cancer, driver oncogenic events promote mutagenesis, and these aberrations are actionable with highly targeted anticancer agents available to effectively modulate driver mutations (2). Increasing knowledge of tumor molecular profiling has led to more sophisticated treatment guidelines, such as those displayed in *Figure 1*. Understanding the molecular profile of tumors can help clinicians decide on the most appropriate treatment course, assist in therapeutic decision making aimed at preventing or overcoming chemoresistance, and ultimately maximize the number of effective treatment

options while minimizing patients' exposure to ineffective, yet toxic, therapies. These potential applications have resulted in a large collaboration, called Lung-MAP, among the National Cancer Institute (NCI), Southwest Oncology Group (SWOG), Friends of Cancer Research, the Foundation for the National Institutes of Health (FNIH), five pharmaceutical companies (Amgen, Genentech, Pfizer, AstraZeneca and MedImmune), and Foundation Medicine. Lung-MAP is a multi-drug, multi-arm, biomarker-driven clinical trial for patients with advanced squamous cell lung cancer (https://clinicaltrials.gov/ct2/show/NCT02154490). Real-time biopsies and diagnostic tests will identify whether patients should receive one of five therapies: an FGFR inhibitor, a PIK3CA inhibitor, a CDK4/6 inhibitor, an EGFR inhibitor, or an anti-PD-L1. A single master protocol can be amended as needed as drugs enter or exit the trial based on efficacy. Collaborative, biomarker-driven clinical trials may prove to be more clinically and cost-effective than traditional large, randomized phase III trials.

The number of pharmacogenetic assays available to identify biomarkers is continuously expanding, with several receiving accelerated FDA clearance and/or approval. The decreasing cost of assays and increasing coverage by third party payers will allow wide accessibility of these assays in clinical practice. While next generation sequencing technologies allow for the identification of a multitude of biomarkers, these technologies are not widely available in the community setting and insurance coverage remains a challenge. However, as the costs of genome sequencing continues to decline to less than $1,000, increasing demand from physicians and patients will shift routine testing from research to clinical practice, in addition to a shift from singleplex testing to multiplex sequencing. As the availability of genomic information and our knowledge of cancer at the molecular level continues to progress, clinicians must understand these intricate molecular pathways, the therapeutic implication of mutations within these pathways, and the clinical assays available to identify such biomarkers.

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None.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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A phase I study of MEDI4736, NNT-PD-L1 antibody in patients with advanced solid tumors

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Abstract: A review and critical consideration of immunotherapeutical concepts in non-small cell lung cancer (NSCLC) is given. Nivolumab represents a promising option in various malignancies with more results exceeding treatment of metastatic malignant melanoma eagerly awaited.

Keywords: Immunotherapy; lung cancer; nivolumab

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Our concept on the emergence and persistence of tumor cells multiplying in an uncontrolled way to become a threatening accumulation of malignant cells visible upon various detection methods has changed quite considerably during the last two decades. Whereas tumors have been primarily thought of as an accumulation of malignant cells proliferating due to molecularly relevant signals to invade vessels and thus spread to distant organs bringing to them malignancy-associated destructive potential, we have recently recognized that tumor cell growth, multiplication and spread is under the influence of the surrounding stroma (1). Tumor cells and the surrounding stroma cells are in close interaction by their *vice versa* influence upon their behaviour by the help of a series of messengers including cytokines, stroma-cell derived products and growth factors for so divergent cellular compounds as endothelial cells, fibroblasts, osteoclasts or immunocompetent cells (1). It is the latter aspect which has generated considerable hope following the discovery and description (2-5) followed by proof of the clinical efficacy of immune checkpoint inhibitors in cancer (6,7). With CTLA4 being the first such immune checkpoint inhibitor and having proved its potential to modulate the disease course in patient with metastatic malignant melanoma (6) and perhaps also in those with non-small lung cancer of squamous cell histology by the anti-CTLA-4 antibody ipililumab (8), the search continued for further compounds targeting tumour-mediated immunosuppression. A major step forward was achieved by the discovery of the induction of T-cell suppression via PD-1 activated by the tumor-cell associated ligand PD-L1 (9). This discovery started the clinical development of antibodies directed against PD-1 or PD-L1 for the use in humans with cancer. The anti-PD-1 antibody nivolumab proved to be effective in patients with malignant melanoma both as monotherapy (10) as well as in combination with ipililumab (11) proving clinically the correctness of the assumption generated by preclinical data.

In the midst of these developments, the study on MEDI4736, an anti-PD-L1 antibody was presented by Lutzky and co-workers at the Annual Meeting of American Society of Clinical Oncology (12). The authors reported on the effect of the human anti-PD-L-1 antibody which prevents binding to PD-1 and CD-80. Within this phase I trial, MEDI4736 was administered i.v. every 2 or 3 weeks in a 3+3 dose escalation in 26 patients with various malignancies. Treatment-related adverse events occurred in 34% of all patients, but—similarly to studies on nivolumab—with a remarkably limited toxicity of grades 1 to 2. Side effects consisted mainly of diarrhoea, fatigue, rash and vomiting. It is noteworthy that particularly autoimmune

phenomena were not induced by the antibody which is in contrast to reports on the toxicity of ipililumab (6). MEDI4736 proved to be clinically effective by inducing four partial remissions and five additional minor responses. These occurred not only in melanoma, but even more so in patients with non-small cell lung cancer (NSCLC) further igniting interest in the use of immune checkpoint inhibitors in this disease with phase III trials on nivolumab in NSCLC are under way and awaited with great interest. Moreover, disease control rate was obtained in almost half of the patients with a durable decrease in tumor size. Thus, the current report corroborates and expands previous observations on the clinical importance of PD-1 and PD-L1 and the therapeutic efficacy of their inhibition. Thus, interventions aiming at a modulation in immune regulation resulting in an increase in T-cell activity by reducing tumor-cell-induced immunosuppression seem more and more to constitute a viable and important concept the results of which will be reported in the very near future and are eagerly awaited. Compounds targeting PD-1 including Nivolumab and Pembrolizumab as well as PD-L1 including MEDI4736, BMS-936559 and MPDL3280A have presented with impressive efficacy and are thus under development in phase I to III studies which will be presented to us in the not too distant future. Thus, the present abstract on the clinical efficacy of MEDI4736 is one more part of the fascinating puzzle successfully linking the immune system to the clinical control of malignant processes.

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Footnote

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Targetting the PD-L1/PD-1 axis holds promise in the treatment of malignancy

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Tumor cells create an immunologic milieu characterized by the disruption of effective antigen presentation, loss of effector cell function and complexity, and upregulation of pathways that promote tolerance and T cell anergy (1,2). A critical element of tumor mediated immunosuppression is the presence of the negative checkpoint molecules CTLA-4 and PDL-1/PD-1 that inhibit immune activation and the expansion of antigen specific T cells (3). In health, these pathways represent an essential stopgap against hyperactivation and the generation of autoimmunity. In the setting of malignancy, upregulation results in an exhausted T cell phenotype that promotes disease growth, resistance to immunotherapy, and disruption of CTL mediated killing of tumor targets (4). Ipilumumab is a clinical grade antibody targeting CTLA-4 that was recently approved as therapy for melanoma (5,6). As a featured theme in the *New England Journal of Medicine*, Brahmer (7) *et al.* and Topolian (8) *et al.* present studies that examine the efficacy of antibody blockade of the PD-1/PDL-1 pathway.

The PD-1/PD-L pathway is an important inhibitory pathway that regulates T cell activation and mediates T cell tolerance. The programmed death (PD)-1 receptor is expressed on T cells, B cells, monocytes, and NKT cells following activation. PD-L1 (B7-H1) and PD-L2 (B7-DC), the two ligands for PD-1, are expressed on antigen presenting cells, including dendritic cells (DCs) and macrophages (4). In addition, PD-L1 is expressed on non-hematopoietic cells including pancreatic islet cells, endothelial cells, and epithelial cells, playing a role in protecting tissue from immune mediated injury (3). Binding of PD-1 to PD-L1 or PD-L2 inhibits T cell proliferation, decreases secretion of Th1 cytokines, and results in T cell apoptosis.

The critical role that PD-1 plays in blunting activated T cell responses was first demonstrated by the autoimmune phenotypes that develop in PD-1 knockout mice (4,9), including cardiomyopathy, diabetes, glomerulonephritis, and arthritis (10-12). It has been shown in models of experimental autoimmune encephalitis that PD-1 blockade exacerbates disease and increases inflammatory infiltrates in the CNS (13,14). In addition, PD-L1 expression on non-hematopoeitic cells, including renal tubular epithelial cells, inhibits immune mediated tissue damage (3,15-17), indicating that the PD-1/PD-L1 pathway is a critical mediator of tissue tolerance. The PD-1/PD-L1 pathway plays an important role in modulating immune response to infection. T cell expression of PD-1 is upregulated during chronic viral infection, resulting in an “exhausted” T cell phenotype. The lymphocytic choriomeningitis virus (LCMV) model was the first to demonstrate the impact of the PD-1/PD-L1 pathway in limiting clearance of virally infected cells (3,18). Barber *et al.* demonstrate that PD-1 expression is upregulated in mice chronically infected with LCMV, and that PD-1 blockade enhanced the clearance of virus (18).

There has been increasing interest in exploring the contribution of the PD-1/PD-L1 pathway to tumor evasion of host immunity. Tumor cells secrete inhibitory cytokines, including TGF-B and IL-10, creating an immunosuppressive milieu and limiting effective anti-tumor immunity. Recent studies suggest that tumor expression of PD-L1 may play an important role in contributing to tumor-mediated immunosuppression. A variety of tumors have been shown to express PD-L1, including renal, melanoma, stomach, breast, and lung carcinoma (19-30). In addition, PD-LI expression on tumor cells has been shown to correlate with

a poor prognosis in a variety of malignancies (19-24,26). In a murine model, it was shown that transgenic expression of PD-L1 rendered a mastocytoma cell line less susceptible to CTL mediated killing and enhanced their tumorigenicity *in vivo*. These effects were reversed in the presence of PD-1 blockade (31). In a melanoma model, PD-1 expression on tumor infiltrating lymphocytes (TIL) was shown to be significantly higher than on T cell isolated from the peripheral blood or normal tissue of the same individuals (32). In this study, PD-1+ TIL demonstrated impaired effector function, as measured by interferon gamma secretion, suggesting that PD-1 expression on TILs limits their capacity to mount an effective immune response. Similarly, Blank *et al.* demonstrated higher levels of PD-1 expression on TIL than on peripheral blood lymphocytes isolated from melanoma patients (12). In addition, PD-1 blockade increased interferon gamma secretion by T cell populations in response to stimulation by antigen loaded dendritic cells. In a muring model of CML, leukemia specific cytotoxic T lymphocytes (CTLs) were shown to express high levels of PD-1, and were functionally exhausted. PD-L1 blockade was shown to restore the function of CML specific CTLs, and prolong survival (32). The effect of PD-1 blockade on enhancing activated anti-tumor T cell responses makes it an ideal therapeutic target to study in the setting of malignancy.

The reports published in the *New England Journal of Medicine* by Brahmer *et al.* (7) and Topalian *et al.* (8), are the first to demonstrate the potency and promise of blocking the PD-1/PD-L1 pathway in the clinical oncology setting. Brahmer *et al.* report on a multicenter phase 1 clinical trial evaluating escalating doses of anti-PD-L1 antibody administered intravenously to patients with advanced malignancies. 207 patients were treated on the study, for a median duration of 12 weeks. Treatment was well tolerated, with treatment related grade 3-4 adverse events occurring in only 9% of patients. The most common treatment related adverse events were fatigue, infusion reactions, diarrhea, arthralgia, rash, nausea, pruritis, and headache. Of note, 39% of patients experienced toxicities thought to be potentially immune mediated including rash, hypothyroidism, hepatitis, and oe case of sarcoidosis, endopthalmitis, diabetes milletus, and myasthenia gravis. These events were predominantly of mild intensity although 9 patients required transient administration of glucocorticoids. Response rates were observed in 9 out of 52 patients with melanoma, 5 of 49 patients with non-small cell lung cancer, 2 of 17 patients with renal cancer, and 1 of 17 patients with ovarian cancer. In addition, prolonged disease stabilization (>24 weeks) was observed in 12-41% of patients with these advanced malignancies.

In the same issue of the New England Journal of Medicine, Topalian *et al.* (8) report the results of a phase 1 multicenter study in which 296 patients with advanced melanoma, non-small cell lung cancer, prostate cancer, or colon cancer were treated with escalating doses of anti-PD-1 antibody. 14% of patients developed grade 3-4 adverse events related to therapy. Common treatment related events include fatigue, rash, diarrhea, pruruutis, decreased appetite and nausea. Toxicities with a potential immune mechanism included pneumonitis, vitiligo, colitis, hepatitis, hypophysitis, and thyroditis. Of particular interest was the presence of pneumonitis occurred in 9 patients that were predominantly responsive to withholding of the drug and/or institution of glucocorticoids, while 3 patients (1%) succumbed as a result of pneumonitis. Responses were seen in 26 of 94 patients with melanoma, 14 of 76 patients with non-small cell lung cancer, 9 of 33 patients with renal cell cancer. Importantly, responses were durable, with 20 of 31 responses lasting a year or greater in patients with at least one year of follow up. Notably, 9 of 25 patients with PD-L1 positive tumors, as asssessed by immunohistochemistical analysis of pre-treatment tumor samples, had disease response. In contrast, none of 17 patients with PD-L1 negative tumors had responsive disease.

The clinical response rates observed in both the Brahmer (7) and Topalian (8) studies validate the role that the PD-1-PD-L1 pathway plays in mediating tumor tolerance, and demonstrates the therapeutic potency of targeting this pathway. Several findings in the studies are of particular note. Responses were observed in non-small cell lung cancer patients, a disease that was classically not thought to be sensitive to immune based treatment. This finding illustrates the potency of immune manipulation, and suggests that immunotherapy may have a role in the treatment of a wide range of tumor types. The durability of responses in both studies was striking. Responses lasting greater than a year in patients with advanced solid tumors are rarely observed in response to chemotherapy or targeted therapy. The potential for inducing durable immune responses that result in long term disease control has the ability to dramatically alter the treatment and natural history of malignancy. Assessing the whether memory T cell responses are induced, determining the optimal treatment duration, and evaluating whether clinical responses are long-lasting, will require further study and longer follow

up. Understanding tumor and host factors that contribute to response to immune based therapy is an area worthy of further study. In the study by Topalian *et al.*, response to anti-PD-1 antibody was associated with tumor expression of PD-L1. Studies to assess tumor markers and host factors that will predict response to treatment are critical to selecting patients who will benefit from immune based therapy. Therapy was generally well tolerated although some significant immune mediated toxicities were observed. Pneumonitis was observed in a small subset of patients patients treated with the PD-1 antibody while inflammatory colitis which has been seen with ipilumumab therapy, was not commonly seen.

The PD-1/PD-L1 pathway is a critical mechanism by which tumors evade immune attack. The clinical studies by Brahmer *et al.* (7) and Topalian *et al.* (8) illustrate the clinical potency of blocking this pathway in the setting of advanced malignancy. The PD-1/PD-L1 pathway has also been shown to be upregulated in response to stimulation with tumor vaccines (33), and studies to evaluate the potential synerygy between tumor vaccines and PD-1 blockade are underway. For example, PD-1 blockade enhances response to the DC/tumor fusion vaccine characterized by polarization of T cells towards a Th1 phenotype, decreased levels of regulatory T cells, and increased CTL mediated killing by fusion stimulated T cells (33). A clinical trial to examining the efficacy of PD-1 blockade in the context of DC/tumor fusion vaccination has been initiated in patients with multiple myeloma undergoing immunotherapy following autologous transplantation. The use of PD-1/PD-L1 blockade alone, and in combination with tumor vaccines, chemotherapy, or targeted therapy, has the potential to dramatically improve outcomes for patients with malignancy. These two clinical trials highlight the therapeutic potency of immune based therapy, and demonstrate that immunotherapy has the potential to alter the treatment paradigm improve outcomes for patients with malignancy.

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Footnote

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Are immune checkpoint blockade monoclonal antibodies active against CNS metastases from NSCLC?—current evidence and future perspectives

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Abstract: Brain metastases occur in approximately half of patients with non-small cell lung cancer (NSCLC) and are associated with a poor prognosis and an inferior quality of life. Historically systemic therapy has had a limited role in CNS disease with a reliance placed on local treatments. The emergence of targeted therapies and immune checkpoint inhibitors (ICIs) in recent years has dramatically changed the treatment landscape of NSCLC. Programmed cell death-1 (PD-1) inhibitors have demonstrated efficacy in three randomized trials and now represent standard second line therapy after platinum failure. Trials have largely excluded patients with symptomatic or untreated CNS disease as the brain has been considered an 'immune-privileged' organ. We review the evidence and future prospects of ICIs in treating brain metastases in NSCLC.

Keywords: Brain metastases; immune checkpoint inhibitors (ICIs); non-small cell lung cancer (NSCLC)

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Introduction

Lung cancer remains the leading cause of cancer mortality in men and women worldwide (1). Non-small cell lung cancer (NSCLC) is the most common subtype accounting for approximately 85% of all lung cancers (2). The 5-year survival in unselected NSCLC at all stages of diagnosis remains less than 20% and for stage IV disease is less than 5% (3,4). In advanced NSCLC, testing for distinct molecular genotypes has led to a personalized approach to treatment, which has improved outcomes when compared to standard platinum chemotherapy (5-13). Maintenance chemotherapy and other targeted agents have had a modest impact on survival (14-16). Immune checkpoint inhibitors (ICIs) are negative regulators of T cells and include anti cytotoxic T-lymphocyte antigen 4 (CTLA-4) antibodies and anti-programmed cell death-1 (anti-PD-1)/programmed cell death receptor ligand-1 (PD-L1) antibodies. These drugs have demonstrated efficacy in NSCLC, melanoma and renal cell cancer, three cancer types with a predilection to brain metastases. Approximately 30–50% of patients with NSCLC can expect to develop CNS disease at some point (17,18). The number of patients with brain metastases is rising and can be explained by the more frequent use of sensitive imaging techniques such as magnetic resonance incidence (MRI) and by the improved survival seen in patients owing to new systemic treatments (19,20). Patients with molecular subtypes such as epidermal growth factor receptor positive (*EGFR*+) and anaplastic lymphoma kinase positive (*ALK*+) lung cancers may have an increased risk of CNS disease at diagnosis compared with *EGFR/ALK* wild-type (WT) NSCLC however this risk may also be explained by a potential lag in diagnosis in this patient population (21-23). The overall survival (OS) in patients with brain metastases is variable and ranges from 3 to 15 months (24). Prognostic

factors such as number of lesions, performance status and extra-cranial control are important determinants (24). In the *EGFR*+ and *ALK*+ subgroups a superior survival of 34 and 38 months respectively has been reported (25).

Historically standard treatments for brain metastases in NSCLC focused on achieving local control with mixed results. Dependent on size, number, symptoms, site and histology of lesions, patients may have been offered surgery and or whole brain radiation (WBRT). WBRT is associated with cognitive decline and inferior quality of life (26-28). While stereotactic radiosurgery (SRS) has the advantage of less cognitive impairment and shorter treatment times, the number of metastases is thought to limit SRS (28). Systemic treatment has inferior CNS disease control due to variable penetration across the blood brain barrier (BBB) (29). Platinum regimens have however demonstrated response rates between 23–50%, which approximated extra-cranial responses (30). Guidelines have suggested that chemotherapy could have a role in patients with asymptomatic disease where local therapies are not possible (31). Bevacizumab in combination with carboplatin/paclitaxel has demonstrated efficacy and early results of a phase II study of 67 patients with non-squamous histology and brain metastases, revealed a 61.2% overall response rate (ORR) in intracranial lesions and a 6-month progression-free survival (PFS) of 56.5% (32). Oral *EGFR*-tyrosine kinase inhibitors (TKIs) and *ALK* inhibitors can gain access to the CNS and response rates, especially in *ALK*+ NSCLC are promising (33-38).

ICIs in NSCLC

The evasion of immune destruction is now recognized as a hallmark of cancer (39). Immune checkpoints are crucial to this and under normal physiological conditions control immune homeostasis and prevent autoimmunity (40). Immune checkpoints belong to a large diverse family of receptors that can negatively impact the efferent immune response by impairing T cell clonal expansion, repressing function and activation and by preventing immune attack against tumor antigens (41). The PD-1/PD-L1 and CTLA-4 axes are the most common checkpoints studied with monoclonal antibodies that can inhibit ligand binding. CTLA-4 is expressed on T cells and appears to primarily inhibit the early activation of effector T cells within lymphoid organs and can enhance the immunosuppressive FOXP3+ regulatory T (Treg) cell population (42). PD-1 counterattacks the T cell response foremost at the tumor or inflammatory site and is upregulated on activated T-cells and other immune cells within the tumor microenvironment. Binding of PD-1 to its ligands (PD-L1 and PD-L2) promotes tumor immune escape by initiating a signaling cascade that inhibits T cell proliferation and limits cytotoxic function (41,43). PD-L1 can be found on a spectrum of cells including endothelial and epithelial cells together with T and B cells, mast and dendritic cells and the high expression of PD-L1 in NSCLC may correlate with inferior prognosis (44). Nivolumab and pembrolizumab are IgG4 monoclonal antibodies targeting PD-1 with early efficacy data presented in phase I studies (45,46). Three large randomized trials have recently confirmed the activity and improved survival of PD-1 inhibitors after failure of first line platinum chemotherapy in unselected NSCLC as well as those selected by tumor PD-L1 expression (47-49). Durable responses across trials are reported in approximately 20% of patients, 30% of those with PD-L1 tumor expression (45,48-50). PD-1 inhibitors now represent a standard option in NSCLC patients with metastatic disease. The efficacy of PD-L1 inhibitors post platinum doublet chemotherapy (POPLAR) and the combination of CTLA-4 inhibitors and PD-L1 inhibitors has also been established (51,52). Trials comparing ICIs to chemotherapy in the first-line setting are expected to report in 2016, with ongoing trials of combination ICI plus chemotherapy regimens versus standard first-line chemotherapy (53,54). The only biomarker known to predict response to PD-1 axis inhibitors in NSCLC is the percentage of PD-L1 positive tumor cells. In KEYNOTE-010, untreated patients who had a tumor proportion score ≥50% (membranous PD-L1 expression in at least 50% of tumor cells) demonstrated higher response rates of 50% (47). This is however far from an ideal biomarker and the lack of PD-L1 expression does not preclude a response (48,49,53,55,56). There has been a growing interest in mutation load as a predictive marker for immune checkpoint inhibition; determining this however, may be costly and impractical on a global scale (57,58). Most of the published studies of ICIs in NSCLC required local CNS control and stability prior to study entry, thus the value of ICIs in patients with brain metastases is understudied.

The immunogenicity of the CNS

Until recently the brain was considered an immune-privileged organ, a term first coined by Billingham and Boswell in the 1950s (59,60). The limited regenerative capacity of neural cells means that strict control must be

in place to prevent autoimmunity. Over the past century foreign tissues and pathogens have been shown to evade the immune system when transplanted into brain parenchyma (61-63). Anatomical barriers such as the BBB and an absent lymphatic system were thought responsible for poor CNS immunogenicity. The latter has now been refuted since the discovery of an intact CNS lymphatic system, which questions our traditional understanding of CSF flow and explains how peripheral immune responses can be generated (64,65). CNS-specific immune cells have also been shown to traverse the cribriform plate in order to reach deep cervical nodes (66). Although the BBB restricts access and flow of peripheral innate and adaptive immune cells, other interfaces such as the CSF and choroid plexus can provide mechanisms of entry (67).

The various compartments of the CNS are complex and heterogeneous in immune cell composition. Microglia are the only immune cells within brain parenchyma and are considered poor antigen presenting cells (68). However within the ventricles, leptomeninges and perivascular spaces are cells of the innate immune system, predominantly macrophages, as well as of the adaptive immune system with a relatively high density of CD4+ memory T cells (67,69). These resident cells are important for ongoing immunesurveillance. Once the CNS becomes inflamed or tumourigenesis initiates, the BBB becomes more permeable and the production of cytokines and chemokines may perpetuate immune cell infiltration (60). Despite this theory, primary CNS tumors do not appear to have a high density of tumor infiltrating lymphocytes (TILs) whereas renal cell carcinomas and melanomas have a higher TIL burden in the microenvironment in CNS metastases (70,71). Similar to systemic disease, the reasons for immune cell heterogeneity within the tumor environment have not been fully explained.

A number of studies have evaluated the prognostic impact of TILs in systemic cancers (72). Within the CNS, the association of TILs with survival has been conflicting. Harter *et al*. investigated a large cohort of patients with CNS tumors including NSCLC metastasis (n=62) and could not find a correlation between TIL burden and patient survival. This group also reported low TIL levels in lung cancer brain metastases, with highest density of TILs in RCC and melanoma (73). Similarly Berghoff reported increased TILs in RCC and melanoma brain metastases but also reported high density in NSCLC samples (n=57), and correlated survival with density of TILs and the 'immunoscore' (71). Both studies were retrospective and the latter only included patients with a single brain metastasis. The median number of lesions in the study by Harter *et al*. was also one. Lung cancer genotype was not available in either study.

An analysis of PD-L1 and TIL densities in NSCLC primary tumor and matched brain metastases revealed higher PD-L1 expression in brain metastases (52% *vs*. 32%) but denser TILs in primary tumors (74). The density of TILs in tumor may be a predictive marker for immune checkpoint inhibition. Given that the non-synonymous mutational burden may represent a predictive marker in NSCLC, the differences in mutational load in systemic disease versus brain metastases may be a contributing factor in TIL differences but this theory has not been explored (57).

Immunotherapy in NSCLC CNS disease—clinical evidence

Clinical evidence to support the efficacy of ICIs in CNS disease is limited. Early data from a phase II study has been reported by Goldberg *et al*. and represents the first report of PD-1 inhibitors in untreated or progressive NSCLC brain metastases (75). This single institution study enrolled 18 patients with melanoma and 18 patients with NSCLC including one *EGFR*+ and one *ALK*+ lung cancer patient. Patients were required to have asymptomatic intracranial disease with at least one brain metastasis measuring between 5 and 20 mm that was untreated. Primary NSCLC tumors had to have at least 1% PD-L1 staining. In the lung group, 10/18 patients had received previous local therapy for brain metastases but evidence of progressive disease. All patients received pembrolizumab 10 mg/kg every 2 weeks until disease progression. Among the patients with NSCLC, 33% of patients (n=6) had a response (four with complete response, one each with confirmed and unconfirmed partial response) with a median response duration of more than 6 months. The numbers of CNS responders in both cohorts correlated with patients achieving a systemic response. Responses in the CNS lasted from 3 to 7 months. It is unknown if responders included specific molecular subtypes. Another third (n=6) of NSCLC patients had confirmed progressive disease intra-cranially and an additional four (22%) could not be evaluated due to rapid systemic progression. The median OS in the NSCLC cohort was 7.7 months but had not been reached in the melanoma group. Neurological toxicities were predominantly grade 1–2, such as seizures, headache and dizziness, and did not result in treatment cessation. Cognitive dysfunction and stroke were less common although a melanoma patient experienced a

Table 1 Ongoing studies including untreated brain metastases in NSCLC

Group or institution trial	Phase	Study	Status
Yale University, NCT02681549	II	Pembrolizumab plus bevacizumab for treatment of brain metastases in metastatic melanoma or NSCLC	Recruiting
BMS, CheckMate 012	I	Study of nivolumab (BMS-936558) in combination with gemcitabine/cisplatin, pemetrexed/cisplatin, carboplatin/paclitaxel, bevacizumab maintenance, erlotinib, ipilimumab or as monotherapy in subjects with stage IIIB/IV NSCLC (CheckMate 012)	Ongoing but not accruing
MD Anderson, NCT02444741	I/II	MK-3475 and hypofractionated stereotactic radiation therapy in patients with NSCLC	Recruiting
Medimmune, D4190C00006	I	A phase Ib study of MEDI4736 in combination with tremelimumab in subjects with advanced NSCLC (52)	Recruiting
AstraZeneca, NCT02179671	II	Immune-modulated study of selected small molecules (gefitinib, AZD9291, or selumetinib + docetaxel) or a 1st immune-mediated therapy (IMT; tremelimumab) with a sequential switch to a 2nd IMT (MEDI4736) in patients with locally advanced or metastatic non-small-cell lung cancer	Completed

NSCLC, non-small cell lung cancer.

transient but severe episode of cognitive dysfunction.

In a phase II study (CheckMate 063) of nivolumab, lung cancer patients with squamous cell cancer who had received at least two lines of systemic treatment were treated with nivolumab. Of two patients with evaluable CNS disease, both had a response (55). Neurotoxicity was again uncommon. A further retrospective review of five patients with NSCLC and new or progressing brain metastases not requiring corticosteroids were treated with nivolumab. Two patients had an intracranial response, including one partial response and one complete response both sustained for over 24 weeks (76). A number of early phase immunotherapy trials are now including patients with untreated asymptomatic CNS disease; however as yet there are no phase III studies that allow enrolment of patients with untreated brain metastases from NSCLC (*Table 1*).

In patients with brain metastases from melanoma, the role of ICIs has been more extensively investigated. Ipilimumab, a CTLA-4 inhibitor, was evaluated in both patients with asymptomatic brain metastases and those with symptomatic disease requiring steroids. The response rates were 18% and 5% respectively (77). It should be noted that 76% of patients with asymptomatic disease had progressive brain metastases at 12 weeks, likely requiring local interventions (78). A retrospective study of ipilimumab reported similar responses (79).

Updated analysis from a phase II study of ipilimumab and fotemustine in metastatic melanoma (NIBIT-M1) has confirmed that 7 of 20 patients enrolled with brain metastases were alive over 2 years from study entry (80). The NIBIT 3 phase III study includes a cohort of patients with untreated asymptomatic brain metastases (81).

Nivolumab has also demonstrated activity in hypermutated glioblastoma and may have a role in primary neurodegenerative disorders such as Alzheimer's disease which reinforces the potential application of ICIs in select populations with intracranial pathology (58,82).

While limited data suggest that intracranial response rates to ICIs are similar to response rates with platinum doublet therapy, ICI therapy has the distinct advantage of producing durable responses in select patients. As yet there is no definitive biomarker to enrich this population. The role of ICIs in *EGFR*+ and *ALK*+ NSCLC has been controversial, with subgroup analyses of phase III trials suggesting no significant survival advantage over second-line chemotherapy (47,48). Gettinger *et al.* on the other hand did report responses in *EGFR*+ patients and a recent study has shown that *EGFR/ALK*+ lung cancer may upregulate PD-L1 expression through activation of PI3K-AKT and MEK-ERK signaling pathways (53,83). In these molecular subgroups where the incidence of brain metastases is high, further clarification of response to ICIs will be important. When brain metastases develop, the cost of patient care rises significantly (84). It is unlikely that use of ICIs without better patient selection will be cost effective in treating an overall poor prognostic cohort of patients.

Future prospects

A number of studies are now investigating the role of

ICIs in patients with untreated brain metastases and it is likely that this will expand following the recent report of Goldberg and colleagues. For example, CheckMate 012, a phase I study of combination nivolumab and ipilimumab in NSCLC, includes an arm of patients with asymptomatic brain metastases (*Table 1*). The role of combination radiation and immunotherapy is a rapidly evolving field. Specifically in the brain metastases population, combinations of ipilimumab/SRS and nivolumab/SRS have demonstrated safety and feasibility in retrospective analyses of melanoma patients (85-87). Kniesley reported a series of melanoma patients with brain metastases and found an improvement in median survival of 21.3 *vs.* 4.9 months when ipilimumab was added to SRS. Radiation necrosis is however, thought to occur with a higher frequency when immunotherapy is used (88). Also the potential for an abscopal effect in malignancy is a subject of great interest, with case reports in NSCLC (89,90). Radiation is thought to repair aberrant vasculature and attract tumor specific T cells into the tumor microenvironment therefore enhancing the immune response (91). Recently it has been shown in mouse models that there is a persistent influx of bone marrow-derived immune cells into the CNS after radiation, suggesting that the physiologic effects of radiation may unleash restraints on the regulation of immune homeostasis (92). The diagnosis of pseudoprogression can be a challenge and case reports of surgical resections have revealed necrotic tissue with inflammatory cells and only scattered tumor cells (93,94).

Given that patients with small asymptomatic brain lesions seem to respond best to ICIs, and that brain metastases have a lower TIL infiltrate compared to primary lung tumors, immunotherapy in the adjuvant setting may be more efficacious in delaying time to development of CNS disease. The adjuvant studies of immunotherapy versus placebo post resection or radical chemoradiation in stage III disease (NCT02273375, NCT02595944, NCT02125461) will help address this question.

Conclusions

A select group of patients with brain metastases from NSCLC may have durable responses to immune checkpoint blockade. More data are needed for better patient selection but this cohort is likely to reflect extra-cranial responders. Combination treatments including radiotherapy may enhance outcomes. In a historically poor prognostic patient population, ICIs offer a promising systemic approach to intracranial disease without major toxicity.

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Footnote

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