Revisiting PD-1 and PD-L1/L2 Pathway in Non-Small cell Lung Cancer– Review

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ABSTRACT
Series of researches done over past few years in oncology have implicated an important role for Programmed death receptor 1 (PD-1) and its ligands Programmed death ligands 1 and 2 (PD-L1 &PD-L2) in immune evasion by a variety of tumors, including lung cancers. PD-L1 (expressed by tumor cells) interacts with the PD-1 receptors on activated T cells, which leads to the inhibition of T-cell mediated immune responses against tumor cells. Thus, targeting this pathway might prove to be an effective method for the development of therapies against lung cancer and improve patient survival. Various Clinical trials are already in progress to evaluate the efficacy and safety of drugs targeting this pathway. Different group of researchers are still trying to establish the precise role of this pathway in tumors like Non-Small cell lung cancer. This review highlights the currently available data regarding the importance of PD-1-PD-L1 pathway in lung cancer (NSCLC) and its current and future implications in lung cancer immunotherapy.

KEY WORDS: Lung cancer, NSCLC, PD-1, PD-L1, PD-L2, immunotherapy.

INTRODUCTION
Lung cancer is one of the most common malignancies worldwide. An estimated 228,190 new cases of lung cancer, accounting for about 14% of cancer diagnoses and 159,480 deaths, accounting for about 27% of all cancer deaths, were expected to occur in USA in the year 2013 1. In 40 European countries lung cancer accounted for 410,000 (12%) of 3.45 million new cancer cases and 20% (353,000) out of 1.75 million cancer-related deaths in 20122. It is also rapidly emerging as a major cause of mortality in the Middle East, Africa, and Asia as well including more than 130,000 lung cancer deaths annually in China 3,4. Death rates attributable to this disease are expected to increase substantially over the next several decades 5,6. Lung cancer’s five-year survival rate (16.3%) is lower than many other leading cancer sites, such colon (65.2%), breast (90.0%) and prostate (99.9%). The five-year survival rate for lung cancer is 52.6 percent for cases with localized (within the lungs) disease although only 15 percent of lung cancer cases are diagnosed at an early stage 7.

Lung carcinomas can be classified as either one of two types: small cell lung carcinoma (SCLC) and Non-SCLC (NSCLC). NSCLC accounts for approximately 85% of all lung cancer and is highly resistant to the conventional treatment modalities. The limited success of conventional therapies, like chemotherapy, radiotherapy and surgery, has led researchers to the development of new therapeutic approaches such as immunotherapy. This approach of modulating the immune system to halt tumor progression has found success to some extent with different tumors. However, various immune escape mechanisms exhibited by the tumor cells are the major obstacle to the success of immunotherapy. Of the various pathways exploited by the tumor to evade the immune system, a
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newly discovered pathway comprising the receptor programmed cell death-1 (PD-1) and its ligand PD-L1 has emerged as an important checkpoint that can be modulated for successful immunotherapy of various tumors4. Importantly, several recent studies have reported successful and durable tumor regression in various tumors induced by blockage of the PD-1/PD-L1 interaction9,10. In this review, we will focus mainly on the expression and role of PD-1/PD-L1 pathway in lung cancer.

**PD-1, PD-L1 and PD-L2 -Role in immune evasion of tumors.**

Various disease processes including tumor progression and metastasis might be modulated by external and internal factors including the host’s immune response. An imbalance in immune regulation affects tumor-specific T-cell immunity in the cancer microenvironment and reshapes the way tumor behaves11. The immune system is kept in check by various immune-inhibitory pathways to prevent the ill consequences of unchecked immune activation such as autoimmune diseases. However, the lack of immune-stimulatory activation may be harmful if it impairs immune responses against different antigens and cancer12. The regulation of T cell expansion and activation is a complex process requiring coordinated interaction of multiple signaling pathways. Several receptor-ligand interactions trigger anti-apoptotic pathways that prevent activation-induced T-cell death13,14. Recently PD-1 has been recognized as an important immune-inhibitory receptor of the CD28 family, which plays a major role in tumor immune escape15,16.

PD-1 is found to be expressed on thymocytes17,18, mature T and B cells following activation19,20 and on myeloid cells18. Compared to the limited expression of CTLA-4, another immune inhibitory receptor of the same type, the wide expression of PD-1 suggests its broader role in immune regulation21. Two ligands for PD-1 (CD279), both belonging to the B7 family, have been identified: PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273)22,23. Interaction of PD-1 with PD-L1, or PD-L2, negatively regulates cytokine production and proliferation of both CD4+ and CD8+ T cells22,24-26. The PD-1/PD-L1 interaction not only inhibits the effector functions (cytotoxicity, cytokine release) of T-cells27 but also induces apoptosis of tumor-specific T cells28, promotes the differentiation of CD4+ T cells into Foxp3 regulatory T cells29 and enhances the resistance of tumor cells to CTL attack30,31. The extent of PD-1 inhibition depends on the strength of the TCR stimulation with more inhibitory effects at low levels of TCR engagement. For instance, CD4+ T cells, inhibition can be overcome by CD28 co-stimulation via IL-2 induction. In contrast, CD8+ T cell inhibition cannot be reversed by CD28 co-stimulation but can be overcome by exogenous IL-235. The similar studies have reported co-stimulatory functions of these ligands, possibly mediated via an unidentified receptor different from PD-132–36. The discordant signaling effects might result from differences in the surface expression of PD-1, a different yet unidentified receptor, or from interfering signals from PD-L2 that can also mediate inhibitory or co-stimulatory effects27,33,35. In addition, PD-1 can induce a state of tolerance on resting dendritic cells (DCs)37.

Though the inhibitory effect of PD-1/PD-L1 pathway has been studied broadly, the molecular mechanism by which PD-1 ligation blocks T cell activation still remains to be clearly defined. However, a few common themes have emerged. First none of these receptors have enzymatic activity, rather their cytoplasmic tails serve as docking stations for signaling proteins that mediate the distinct effects of each of these molecules38. Second, the phosphorylation status of the cytoplasmic tails of CD28 family members influence which proteins are recruited. PD-1 contains two tyrosine molecules within its cytoplasmic tail. The most membrane-proximal tyrosine is located in an ITIM, and the distal tyrosine is located in an immune receptor tyrosine-based switch motif (ITSM), a stretch of amino acids that was recently identified by virtue of its ability to bind to the small adaptor Src homology 2 domain containing molecule 1A (SH2D1A)39like the B cell receptor, CD40, and CD95, can transmit positive or negative signals. CD150 can associate with the SH2-containing inositol phosphatase (SHIP. The study done by Jens M. Chemnitz and colleagues identified that the mutation of the ITIM was found to have little effect on PD-1 signaling or functional activity. In contrast, even a single mutation of the ITSM abolished the ability of PD-1 to block cytokine synthesis and to limit T cell expansion. This result indicated a second explanation for discordant results after PD-L1/PD-1 interaction. Their results also showed that the ITSM of PD-1 fails to recruit SH2D1A, which sets a basis that PD-1 signaling mechanism might be different than other receptors.
containing ITSM. The PD-1 signaling might depend on the activation status of the T cell and close proximity of PD-1 with CD3 and/or CD28 may be necessary for it to act as inhibitor of T cell activation. We constructed a chimeric molecule consisting of the murine CD28 extracellular domain and human PD-1 cytoplasmic tail. When introduced into CD4 T cells, this construct mimics the activity of endogenous PD-1 in terms of its ability to suppress T cell expansion and cytokine production. The cytoplasmic tail of PD-1 contains two structural motifs, an ITIM and an immunoreceptor tyrosine-based switch motif (ITSM).

The patterns of expression of PD-L1 and PD-L2 are quite distinct. PD-L1 is constitutively expressed by a wide variety of immune cells and non-immune cells and most normal tissue cells seem to be able to up-regulate PD-L1 in the presence of strong inflammatory signals, likely to prevent collateral damage induced by potent and potentially destructive Th1/17 T-cell responses. In comparison, constitutive basal expression of PD-L2 is low. Initially PL-L2 expression was thought to be restricted to antigen-presenting cells such as macrophages and DCs. In recent years, we constructed a chimeric molecule consisting of the murine CD28 extracellular domain and human PD-1 cytoplasmic tail. When introduced into CD4 T cells, this construct mimics the activity of endogenous PD-1 in terms of its ability to suppress T cell expansion and cytokine production. The cytoplasmic tail of PD-1 contains two structural motifs, an ITIM and an immunoreceptor tyrosine-based switch motif (ITSM).

PD-L1 surface expression can be found on almost all tumor entities including the surface of human cancers of larynx, lung, stomach, colon, breast, cervix, ovary, renal cell, bladder, liver, glioma and melanoma. A member of the CD28/CTLA4 family expressed on activated lymphoid cells. PD-1 contains an immunoreceptor tyrosine-based inhibitory motif and mice deficient in PD-1 develop autoimmune disorders suggesting a defect in peripheral tolerance. Human PD-L1 and PD-L2 are expressed on immature dendritic cells (iDC). In vitro experiments indicate that many tumor cell lines also express PD-L1, and/or up-regulate PD-L1 surface expression, upon exposure to IFN-γ and PD-1(-/-). In vitro experiments using PD-L1 over-expressing murine tumor cell lines, and blocking antibodies against PD-L1 and PD-1 clearly demonstrated that PD-L1 on tumor cells suppressed the cytolytic activity of CD8+ T cells.

In addition, researchers showed that endogenous PD-L1 expression on tumor cells was also capable of suppressing T-cell functions including proliferation and cytokine production. In vivo experiments using blocking antibodies of PD-1, PD-L1 or TCR-transgenic mice crossed to PD-1(-/-) mice revealed accelerated tumor eradication in the absence of PD-L1/PD-1 interaction. In addition, PD-L1 blockade suppresses tumor metastasis in melanoma, or colon cancer, cell lines. The correlation between cancer patient’s survival and expression of PD-L1 in tumor cells or stromal fibroblasts have been studied in various cancers like melanoma, renal cell carcinoma, breast cancer etc. with almost all studies showing the negative impact of increased PD-L1 expression on overall survival. However, recently few researches published have shown results contradictory to the past work, identifying PD-L1 as a positive prognostic factor for overall survival in various tumors like colon carcinoma, merkel cell carcinoma as well as NSCLC.

**PD-L1/PD L2 expression and lung cancer- Clinical correlation**

Though previously thought to be non-immunogenic, lung cancer exhibits various immune escape mechanisms, such as the expression of negative regulators, to survive immune attack. To date several studies have tried to establish a co-relation between PD-L1/PD-L2 expression in lung cancer cells and the survival outcome of the patients. In 2004, Jun Konishi et.al investigated the expression of PD-L1 (B7-H1) and PD-L2 (B7- DC) in tumor specimens of NSCLC and its relationship with clinico-pathological variables and post-operative survival and did not find any relationship between the expression of these ligands on tumor cells and those variables. However, they found that the expression of B7-H1 on cells residing in local areas reciprocally correlated with number of TILs (tumor infiltrating lymphocytes) and may indeed contribute to the negative regulation in anti-tumor response in NSCLC. However, in another study where immune-histochemical analysis was used to evaluate the expression of PD-L1 in 109 non-small cell lung cancer (NSCLC) tissues and para-tumor tissues, researchers found that the expression rate of PD-L1 was associated with histological types and overall survival. Patients with adenocarcinoma or with a survival time post-surgery of less than 3 years showed...
higher expression of PD-L1. Yet another group of researchers investigated the level of soluble PD-L1 in peripheral blood of lung cancer patients and found that the elevated expression of sPD-L1 in lung cancer patients was closely related to lung cancer staging, metastasis and clinical response. A 5-year follow up study conducted by Chen YB, Mu CY, and Huang J A in NSCLC patients reported that no PD-L1 was detected in benign lung tumors whereas 57.5% of NSCLC tissue specimens showed PD-L1 expression. They found no relationship between PD-L1 expression and patient age, gender or histo-pathological type. However, PD-L1 expression was found to be significantly correlated to the degree of tumor cell differentiation, stage of Tumor Node Metastasis (TNM) and patient survival.

They showed that PD-L1 status was a significant independent prognostic factor of NSCLC. In one of the studies where PD-L1 mRNA expression was examined in NSCLC tissue and paratumor tissue, researchers found that the T/N ratio of PD-L1/β-actin mRNA level showed a gradual increase in pathological T stages, and was markedly higher in pathological T4 cases, but there was no statistically significant difference on the mRNA levels of PD-L1/β-actin in lung cancer and adjacent normal lung tissues, or a correlation of the T/N (tumor /normal tissue) ratio of PD-L1/β-actin mRNA levels with gender, age, EGFR mutations status, pathological subtypes and lymph node metastasis. In concise, all of these studies in the past identified PD-L1 expression by tumor as negative prognostic factor in NSCLC patients by virtue of their ability to inhibit T-cell mediated immunity. This in turn arouse an immense enthusiasm in the field of immunotherapy leading to the development of drugs targeting this pathway.

However, the reports of large scale studies published recently have once again challenged the results of previous researches. In one of these research, increased PD-L1 has been reported in cases of sarcomatoid carcinomas (SCs) of the lung; a rare and aggressive type of NSCLC, in much higher level than conventional NSCLC. The results of this study were concordant to another large study where two cohorts of NSCLC cases including 340 cases from hospitals in Greece and 204 cases from Yale University were assessed by using various antibodies and in situ mRNA hybridization techniques were used to measure PD-L1 in non-small cell lung cancer (NSCLC) by quantitative fluorescence (QIF) approach. In these experiments, PD-L1 expression was found to be significantly associated with tumor-infiltrating lymphocytes and patients with PD-L1 (both protein and mRNA) expression above the detection threshold showed statistically significant better outcome in both series independent of histology. These results are consistent with two recent studies, one in Merkel-cell carcinomas, and other in colorectal carcinoma showing association of PD-L1 protein expression with increased TILs and longer survival. These new findings suggest that PD-L1 might act as a marker for inflammatory process going on in the tumor microenvironment, and also emphasize the importance of understanding the effect of other immune regulatory pathways on PD-1-PD-L1 pathway.

These conflict in results of different research might be due to the difference in material used (formalin fixed, paraffin embedded tissue versus fresh frozen tissue), variation in sample size, methods of identification and quantification of PD-L1 and PD-L2 and the primary antibody used and observer variations. The role of PD-L1 expressed in cytoplasm of tumor cells has not yet been fully understood and most of these past researches had taken in account expression of PD-L1 by cytoplasm as well as plasma membrane while exploring its significance in NSCLC. Larger number of recent studies have considered expression of PD-L1 in cell membrane of at least five percent of tumor cells as cut-off point to divide its expression as positive and negative. These methodological variances certainly have great impact on the results and have been a cause of dilemma to researchers. Also one important drawback of these studies are that these were all retrospective studies done on tissue samples usually obtained and prepared long before the experiment. So, the heterogeneity in results might have occurred because of treatment effect, loss of specific epitope in sample as a result of tissue storage and processing. Therefore, standardization of the methods and materials for identification and quantification of these important ligands should be done to reduce procedure related variation in results. As, PD-L1 is being considered as one of the prognostic markers in NSCLC, it is very important to develop a tool to measure the level of PD-L1 and generate the results that are accurate and reproducible.

Although there is no correlation between the expression of PD-L2 in tumors and clinical outcome, Nazareth and colleagues found constitutively high
PD-L1 and PD-L2 expression in fibroblasts that were cultured from human NSCLC. This expression appeared to be functional since in vitro blocking studies demonstrated that the fibroblasts inhibited IFN-γ production by autologous T cells in a PD-L1- and 2-dependent manner. For this reason, future studies should not only focus on PD-L1 and PD-L2 expression by tumor cells only but also by the tumor stroma. These studies further support a role for the PD-L1/ PD-1 pathway in subversion of tumor immunity and point to PD-L1 expression as a useful biomarker to identify patients at greater risk of metastatic disease progression and death. Moreover, this knowledge supports the development of therapies to block this pathway in order to arouse seemingly dormant immune responses against cancers.

**Blocking PD-1/ PD-L1 pathway for lung cancer therapy**

A major implication of the clinical activity of an immune checkpoint blockade is the generation of endogenous immune responses to tumor antigens that can be harnessed therapeutically to induce tumor regression. An emerging concept in cancer immunology is that inhibitory ligands such as PD-L1 are induced in response to immune attack, a mechanism termed adaptive resistance. There are currently six agents blocking the PD-1/PD-L1 pathway in clinical evaluation. They are: MDX-1106/BMS-936558/ONO-4538 (fully human IgG4 anti-PD1 mAb from BMS), CT-011 (humanized IgG1 anti-PD1 mAb from CureTech/Teva), MK-3475 (human IgG4 anti-PD1 mAb from Merck), MPDL3280A/ RG7446 (anti-PD-L1 from Genentech), BMS-936559 (fully humanized PD- L1lgG4 mAb inhibiting ligation to both PD-1 and B7.1) and AMP-224 (a B7-DC/IgG1 fusion protein licensed to GSK) (http://www.clinical trials.gov).

In the study reported by Brahmer et al. in 207 patients (with different tumors of which 75 were lung cancer) using the BMS-936559 (anti-PD-L1 mAb) in a multicenter Phase 1 trial at multiple escalating doses (from 0.3 to 10 mg/kg). In patients with NSCLC, there were five objective responses (in four patients with the non-squamous subtype and one with the squamous subtype) with response rates of 8% and 16%, respectively. They reported in their study that the objective response in 5 of 49 patients (10%) with advanced non–small-cell lung cancer to be of great significance as it was always considered to be non-immunogenic. In another Phase 1 trial, conducted by Topalin and team which included 122 NSCLC cases along with other tumors to assess the safety, anti-tumor activity, and pharmacokinetics of BMS-936558, a fully human IgG4-blocking monoclonal antibody directed against PD-1 at multiple escalating doses (from 0.3 to 10 mg/kg). Objective responses were observed across non–small-cell histologic types: in 6 out of 18 patients (33%) with squamous tumors, 7 out of 56 (12%) with non-squamous tumors, and 1 out of 2 with tumors of unknown type. According to the study, an objective response in 36% of the patients with PD-L1–positive tumors and no response in those with PD-L1–negative tumors suggests that PD-L1 expression on the surface of tumor cells in pretreatment tumor specimens may be associated with an objective response. Another notable observation was that not all those patients who had PD-L1 positive tumors were responsive to anti-PD-L1 therapy, indicating a need for personalized medicine according to each patient’s need and specific markers.

These studies showed slight differences in the differential efficacy of both agents, in favor of the anti-PD-1 mAb, which can block the engagement of PD1 by both PD-L1 and PD-L2 and a less toxic effect than anti-PD-L1. They describe clinical activity and safety of these agents and validate the importance of the PD-1–PD-L1 pathway for the treatment of some cancers including the lung cancer. Phase 2 trials involving immunologic and molecular-marker correlates (ClinicalTrials.gov numbers, NCT01354431 and NCT01358721) are under way, and phase 3 studies of anti–PD-1 antibody for the treatment of non–small-cell lung cancer, melanoma, and adrenal-cell cancer are being planned. Out of many new potential drugs targeting these pathways which are being studied, FDA has recently approved immunotherapy nivolumab (BMS-936558) for the treatment of advanced (metastatic) squamous NSCLC that has failed chemotherapy. This approval was based on results of a phase III trial which showed that patients receiving nivolumab lived, on average, 3.2 months longer than patients receiving standard chemotherapy, which translates into a 40% reduced risk of death.
CONCLUSION

Various studies done in the field of cancer immunotherapy have established the importance of PD-1/PD-L1 pathway in immune escape of various tumors including human lung cancer. Although lung cancer has long been considered non-immunogenic, recent studies showing the role of various immune escape mechanism in lung cancer progression have changed dramatically the outlook about therapeutic options in lung cancer. Different immunotherapies are being considered for the treatment of lung cancers, including NSCLC, which has a very poor prognosis. Results of phase-1 studies involving anti PD-1 and anti PD-L1 are very promising and encouraging to develop these as new treatment modalities for lung cancer. Yet, the results of various studies are still conflicting with some earlier ones identifying PD-L1 as negative predictive marker and the latest researches comprising larger cohort of patients showing PD-L1 associated with tumor as a positive predictive marker of survival. More of larger scale studies with standardized technique and materials to identify as well as quantify PD-L1 associated with clinical outcome are required to identify the potential role of PD-L1 in different histological types and grades of lung cancer in different populations. Further researches and clinical trials are also required to establish a proper treatment regimen according to each patients need (personalized patient care) depending on specific markers. Potential benefit of Combination therapies with different checkpoint blockers should be explored. Moreover, the molecular mechanisms of the PD-1/PD-L1 pathways and also its agonist and antagonistic pathways should also be explored further to fully understand the role of this pathway in progression and metastasis of NSCLC. This will provide the rationale to support new hypothesis driven studies and pave a way towards finding a cure for NSCLC.

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