Journal of
Clinical & Cellular Immunology

Yi, J Clin Cell Immunol 2014, 5:6
DOI: 10.4172/2155-9899.1000274

Review Article Open Access

Immune Microenvironment and its Therapeutic Implication in Non-Small Cell Lung Carcinoma: Literature Review

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Received date: August 04, 2014, **Accepted date:** November 15, 2014, **Published date:** November 24, 2014

Abstract

Due to the genetic and epigenetic alterations, cancer cells may express antigens that can trigger host immune responses. T cells play a major role in the host immune response, which is initiated via antigen recognition by T cell receptors and regulated by a dynamic balance between co-stimulatory and inhibitory signals, also known as immune checkpoints. While immune checkpoints are crucial for the prevention of autoimmunity by maintaining self-tolerance, tumor cells can exploit these pathways to forge a suppressive immune microenvironment for preventing tumor cell destruction. Recently, several immune checkpoint modulators have been tested for treating non-small cell lung carcinomas and some of them offered very promising results. More studies are needed in the area of biomarker testing for selecting the patients who will respond to these immunotherapeutic agents.

Keywords: CTLA-4; PD-1; PD-L1; TIL; Non-small cell lung carcinoma; Immune check points; Immune microenvironment

Abbreviations

APC: Antigen Presenting Cell; CTLA-4: Cytotoxic T-Lymphocyte-Associated Antigen 4; GITR: Glucocorticoid-Induced TNF Receptor; IFN-γ: Interferon Gamma; KIR: Killer Cell Immunoglobulin-Like Receptor; LAG3: Lymphocyte-Activating Gene 3; mAb: Monoclonal Antibody; NSCLC: Non-Small Cell Lung Carcinoma; PD-1: Programmed Cell Death 1; PD-L1: Programmed Cell Death Ligand 1; TCR: T Cell Receptor: TIL: Tumor Infiltrating Lymphocyte; TIM3: T-Cell Immunoglobulin- And Mucin Domain-3-Containing Molecule 3; TNF: Tumor Necrosis Factor; TMA: Tissue Microarrays

Introduction

Lung cancer is the leading cause of cancer death worldwide for both men and women. NSCLC refers to a heterogeneous group of lung carcinomas including the 3 main types: adenocarcinoma, squamous cell carcinoma and large cell carcinoma. NSCLC comprises 75% to 80% of all lung cancers and currently available conventional chemotherapeutic regimens have done relatively little to improve outcome in NSCLC patients over the past decades. However, recent advances in targeted therapies against the oncogenic driver mutations have improved treatment outcomes in lung cancer patients, particularly who have adenocarcinomas with appropriate molecular targets in their tumors. Despite these advances in targeted therapy, most patients who responded initially will eventually develop resistance and overall survival of patients with NSCLC still remains dismal. Also, these targeted therapies are mostly for adenocarcinomas occurring in nonsmokers, but not for squamous cell carcinomas that primarily affect smokers.

Recently, it has been noted that interactions of cancer cells with the host immune system are as important as the properties of cancer cells per se in tumorigenesis [1]. Smoking- and pollution-associated lung cancers possess a high density of missense mutations in expressed genes [2,3]. These genetic alterations, together with other changes via epigenetic dysregulation, provide lung cancer cells with many tumor specific neoantigens recognizable by host T cells. Tumors can evade immune destruction via immune checkpoint signals that suppress the host immune responses to tumor cells. A better understanding of tumor immunosurveillance led to the development of a new generation of immunotherapeutic agents that restore the host immune response to tumor cell antigens. Several immunotherapeutic agents have been tested in clinical trials. Results from early phase studies of these immune checkpoint modulators are highly promising in NSCLCs. This new approach may prove to be effective especially in the treatment of squamous cell carcinomas, for which there is no currently available FDA-approved targeted therapy.

Major questions and challenges in immunotherapies for NSCLC have been identified and these will need to be addressed. More work is needed to establish an optimum schedule for incorporating immunotherapeutic agents with other treatments (such as cytotoxic chemotherapy, molecular targeted therapy and radiotherapy). The potential benefit of immunotherapy in treating early stage disease or as a first line therapy needs to be explored as well. Also, biomarkers for predicting the responsiveness to immunotherapy have yet to be determined in NSCLC. In this review, several issues relevant to immunotherapy in NSCLC will be discussed based on the current literature organized under the following headings: Tumor microenvironment; Tumor infiltrating lymphocytes (given their major role for the immune microenvironment of tumors); Immunotherapeutic agents for NSCLC (that are under clinical trials); Biomarker testing for immunotherapies.

Tumor Microenvironment

Tumor microenvironment is comprised of dynamic balances caused by interactions between the stimulatory or inhibitory receptors in the immune cells (including lymphocytes and various antigen presenting histiocytes), and their corresponding ligands present in the

tumor cells as well as in some of the immune cells. Cancer cells may express antigens that differ from those of normal host cells due to genetic and epigenetic alterations occurring during carcinogenesis [4]. To eliminate the cancer cells, the host immune system must recognize the tumor antigen first, and then present it to T cell receptor, which will lead to T cell activation and killing of the cancer cells. Several co-stimulatory and inhibitory receptor molecules are normally present on the surface of immune effector cells to regulate the T-cell mediated immune response. Known co-stimulatory receptors include CD27, CD28, CD40, CD137, GITR, and OX-40 (CD134) that will promote the T-cell response [5-15]. Conversely, co-inhibitory receptors, including CTLA-4 (CD152), PD-1 (CD279), TIM3, LAG3 (CD223) and KIR, prevent immune response in order to protect against autoimmunity in physiologic status [1,16-23]. For each of these stimulatory and inhibitory receptors, there are specific corresponding ligands that are normally present not only on antigen presenting cells (APCs) and other immune cells, but also on non-immune cells including tumor cells or some normal cells of solid organs [1]. Binding of inhibitory receptors on immune effector cells and their ligands on APCs, tumor cells, or non-immune normal cells can lead to immune tolerance: i.e. immune checkpoint. Cancer cells use these immune checkpoints to escape host immune system by creating an immunosuppressive microenvironment that down regulates T-cell activation and cell signaling. Immune checkpoint blocking agents disrupt this immune resistant mechanism by the tumor cells and establish a durable tumor control [24].

It has been postulated that potential antigenic targets in lung cancer can escape immune rejection by two very different mechanisms: 1. "editing" out particularly immunogenic neoepitopes, 2. induction of antigen-specific tolerance [25-27]. Editing implies that T-cell recognition of a tumor neoantigen results in the selection of tumor cells lacking antigen, whereas tolerance induction implies that tumor specific T cells become incapable of attacking antigen-bearing cells. The relative importance of editing versus tolerance induction in human lung cancer remains to be determined [2].

T-cell mediated immunity includes multiple sequential steps involving the clonal selection of antigen specific cells, their activation and proliferation in secondary lymphoid tissues, their trafficking to the sites of antigen and inflammation, the execution of direct effector functions and the provision of help through cytokines and membrane ligands for a multitude of effector immune cells [28]. Each of these steps is regulated by counterbalancing stimulatory and inhibitory signals that fine tune the response. Although virtually all inhibitory signals in the immune response ultimately affect intracellular signaling pathways, many are initiated through membrane receptors, the ligands of which are either membrane bound or soluble (cytokine). Restifo et al. [29] showed that suppressed antigen presenting molecules could be upregulated by IFNγ in the majority of lung cancer cell lines. This finding is highly relevant to immunotherapy because it suggests that suppression of tumor antigen presentation can be reversed in the majority of lung cancers if T cells or NK cells, the two major producers of IFNγ, could be activated within the tumor microenvironment.

PD-1 is expressed on activated T-and B-cells and its major ligand PD-L1 (B7-H1) is typically expressed on the subset of macrophages, but can be induced by inflammatory cytokines in a variety of tissue types [30]. When activated T-cells expressing PD-1 encounter PD-L1, T-cell effector functions are diminished. PD-1 also binds PD-L2 (B7-DC), which is expressed selectively on macrophages and dendritic cells [31]. These unique expression patterns suggest that PD-L1 promotes self-tolerance in peripheral tissues, while PD-L2 may function in lymphoid organs, although the role of PD-L2 in immunomodulation is not as well understood. Shi et al. reported that PD-L2 protein is robustly expressed by the majority of primary mediastinal large B-cell lymphoma, but only rare diffuse large B-cell lymphomas and is often associated with *PDCD1LG2* copy gain [32].

In NSCLC, the most therapeutically relevant mechanism for immune resistance might be the expression of immune inhibitory receptor molecules in the tumor microenvironment. These molecules fall into a number of classes based on the nature of inhibitory ligand: cytokines, membrane ligands, and metabolites [2]. The two inhibitory cytokines commonly expressed in lung cancers are IL-10 and TGF-β. Among the membrane inhibitory ligands, PD-L1 has been the most studied in NSCLC, though PD-L2, B7-H3, and B7-H4 have also been reported as upregulated in lung cancer [28,33]. PD-L1 is expressed on tumor cells in approximately half of NSCLC but is sometimes expressed on myeloid cells in the stroma surrounding tumor cell nests.

Though not often considered, immune inhibitory metabolites are probably also important players in local immune resistance in lung cancer. Concentrations of adenosine, which binds to the inhibitory G-protein-coupled A2a receptor (A2aR) expressed on lymphocytes, have been shown to be extremely high in NSCLC tissue. Triggering of A2aR inhibits effector T-cell function and drives the development of Tregs, another inhibitory component of the tumor microenvironment [34,35].

As a general rule, the stimulatory or inhibitory receptors and their ligands implicated in T cell activation are not necessarily overexpressed in cancers as compared to normal tissues. On the other hand, the inhibitory receptors and ligands that regulate T-cell effector functions are commonly overexpressed on tumor cells or on non-transformed cells in the tumor microenvironment [28]. The soluble and membrane-bound receptor-ligand immune checkpoints have been found to be the most amenable to potential therapies via blocking antibodies for inhibitory pathways. Thus, unlike the other currently used antibodies for cancer therapy, the antibodies that block immune checkpoints do not necessarily target tumor cells. Rather, they mainly target the inhibitory receptors on the immune cell surface or their ligands in order to enhance endogenous antitumor activity.

Tumor Infiltrating Lymphocytes

Tumor Infiltrating Lymphocytes (TILs) refer to the lymphocytes within and around the tumor cells. Interplay between tumor cells, stromal cells, and host inflammatory cells has been shown to be an important factor in the development of malignant tumor [36]. The presence of TILs within the tumor microenvironment is considered to be an indication of the host immune response to tumor antigens and is thought to reflect the dynamic processes of cancer immunoediting [36]. A previous study suggested that immune infiltrates within the primary tumors and metastatic sites might be independent prognostic biomarkers [37].

Increased numbers of TILs comprised of CD4+ and CD8+ T-cells have been reported to be a good prognostic factor in lung cancer, whereas increased numbers of TIL with Foxp3+ T-cells have been reported to be a poor prognostic factor [38-40]. Kilic et al. reported that a higher degree of TILs within a large node-negative NSCLC correlated with decreased risk of disease recurrence and improved disease-free survival in 219 lobectomy cases [41]. These are, however, relatively crude analyses and much more work needs to be done to

assess the biologic and therapeutic relevance of expression patterns of multiple inhibitory ligands as well as the distribution and expression pattern of their corresponding receptors on TILs. Gerdes et al. [42] reported a novel method of highly multiplexed single-cell analysis of formalin-fixed, paraffin-embedded cancer tissue. They used a multiplexed fluorescence microscopy method for quantitative, single-cell, and subcellular characterization of multiple analytes. Chemical inactivation of fluorescent dyes after each image acquisition round allows reuse of common dyes in iterative staining and imaging cycles. This new technique could be potentially useful to assess the immunologic milieu of tumor, by examining multiple markers at the single cell level.

The role of tumor infiltrating B-cells is not well known. Recently, Germain et al. demonstrated that B cells organized into tertiary lymphoid structures (encompassing follicular B-cells, clusters of mature dendritic cells and T-cells) exhibit features of an ongoing humoral immune response, and that their high density is associated with the long-term survival of patients with NSCLC [43]. The presence of both types of APCs including mature dendritic cells and B-cells in tertiary lymphoid structures predicted a good outcome of the patients; a low density of both follicular B cells and mature dendritic cells correlated with poor survival of NSCLC patients. In this study, the organization of intratumoral B cells into B-cell follicles was associated with the development of antigen-specific humoral responses, with the emergence of plasma cells secreting tumor antigen-specific immunoglobulins, which might lead to the identification of new therapeutic targets in NSCLC. They concluded that B cell density may represent a new prognostic biomarker for NSCLC patient survival, and they also made the link between tertiary lymphoid structures and a protective B cell-mediated immunity.

The predictive significance of TILs for response to neoadjuvant chemotherapy in NSCLC is not well known. Liu et al. tested a hypothesis that the presence of lymphocytic infiltration in tumor tissue may predict the prognosis and response to chemotherapy. The aim of this study was to investigate the prognostic and predictive value of TIL subtypes in patients with advanced NSCLC when treated with platinum-based chemotherapy. They evaluated the distribution and densities of TIL subsets in paraffin embedded tumor tissues from 159 patients with stage III and IV NSCLC and correlated with the clinicopathologic parameters [37]. They also investigated the predictive values of the type, density and combination of TILs for overall survival and chemotherapeutic responsiveness in their NSCLC cohort. The prevalence of CD3+, CD4+, CD8+ and Foxp3+ TILs was assessed by IHC in tumor tissue obtained before chemotherapy. The presence of CD3+, CD4+, CD8+ and Foxp3+ TILs did not correlate with any clinicopathological features in this study. Neither the prevalence of TILs nor the combined analysis displayed obvious prognostic performances for overall survival. However, higher Foxp3+/CD8+ cell ratio in tumor sites was an independent factor for poor response to platinum-based chemotherapy in overall cohort. These findings suggested that CD8+ and Foxp3+ Tregs cell infiltrate within tumor microenvironment may be predictive of response to platinum-based neoadjuvant chemotherapy in advanced NSCLC patients.

Immunotherapeutic Agents for NSCLC

Cancer immunotherapy uses the immune system to treat cancer by provoking the host immune system into attacking the tumor cells via cancer antigens as targets. A major turning point in cancer immunotherapy came with the clinical application of antibodies that block immune checkpoints related to the immune inhibitory ligand-receptor interactions in the tumor microenvironment. The two immune checkpoint receptors CTLA-4 and PD-1 have been most actively studied in this context [6,44-49]. They regulate immune responses at different levels and by different mechanisms; CTLA-4 counterbalances the co-stimulatory signals delivered during T-cell activation, whereas PD-1 predominantly down regulates T-cell responses in tissues. Given the clinical benefit of the antibody blocking either of these receptors, antitumor immunity can be enhanced at multiple levels and a combination therapy might allow synergistic effects in the treatment of NSCLC. In the PD-1 pathway, PD-1 and one of its ligands PD-L1 have been blocked. The PD-1 ligands PD-L1 and PD-L2 are induced by distinct inflammatory cytokines. PD-L1 expression can be induced on diverse epithelial and hematopoietic cell types, while PD-L2 is predominantly expressed on dendritic cells and macrophages as mentioned earlier.

Multiple tumor types have been shown to express PD-L1 and PD-L2, effectively co-opting a native tolerance mechanism. The more selective expression pattern of the ligands for PD-1, as compared to the ligands for CTLA-4, has important treatment implications. First, it suggests that more focal immune-related side effects may be encountered with PD-1 blockade compared to CTLA-4 blockade, which has been also predicted by the fact that murine CTLA-4 knockout model turns out to be a fatal phenotype. Second, it suggests that the local tumor microenvironment may be the key site to yield evidence of molecular markers predicting clinical response to PD-1 pathway blockade.

CTLA-4 Inhibitors

Ipilimumab, a monoclonal antibody (mAb) against CTLA-4, is the prototype drug directed against an immune checkpoint. It blocks the co-inhibitory CTLA-4 receptor on T-cells from interacting with its ligands, B7-1 and B7-2 that are expressed on APCs but not on solid tumors. Ipilimumab was the first drug to demonstrate increased overall survival in patients with advanced melanoma, but has shown virtually no effect as a single agent in treating lung cancers [2]. However, modest benefit has been shown in combination with standard chemotherapy using paclitaxel and carboplatin, especially in NSCLC patients with squamous cell histology.

PD-1 Inhibitors

Blocking antibodies against the receptor PD-1 have shown significant antitumor activity as single agent in advanced NSCLC, in contrast to anti-CTLA 4 antibody that has been ineffective as a single agent. Nivolumab (BMS-936558/MDX-1106/ONO-4538) and Lambrolizumab (MK3475) are anti-PD-1 antibodies that block the binding of PD-1 receptor to its two ligands PD-L1 (B7-H1) and PD-L2 (B7-DC). Antitumor activity and safety of Nivolumab has been studied in a total of 296 patients including advanced melanoma, NSCLC, castration resistant prostate cancer, renal cell and colorectal cancers. This anti-PD-1 antibody produced objective responses in approximately one in 4 to one in 5 patients with NSCLC, melanoma, or renal cell cancer [50]. Taube et al. reported that approximately 30% of patients with treatment-refractory advanced melanoma and renal cell carcinoma receiving Nivolumab experienced durable objective tumor regressions [51]. Objective tumor responses were also observed in 17% of patents with NSCLC. In addition to the significant response and disease stabilization rate in advanced chemotherapy refractory

patients, the durability of responses were remarkable; median duration of response was 74 weeks, as compared with 4-8 months for chemotherapy regimens and oncogene targeted tyrosine kinase inhibitors. The encouraging results of the reported clinical trials have led to additional phase III trials of Nivolumab as single agent compared with docetaxel. Multiple treatment arm phase I trials in NSCLC combining Nivolumab with various chemotherapy regimens as well as with Ipilimumab, an anti-CTLA-4 mAb, have been launched. Lambrolizumab, another anti-PD-1 mAb, has been also tested in NSCLC with results pending.

PD-L1 Inhibitors

Three anti-PD-L1 antibodies including BMS-936559, MPDL3280A, and MedI-4736, have been evaluated or are being evaluated in NSCLC. There has been much debate about the relative merits of antibodies directed against PD-1 versus PD-L1. Anti PD-1 antibodies block its binding to both known PD-1 ligands, PD-L1 and PD-L2, whereas anti-PD-L1 antibodies only block the PD-1: PD-L1 interaction. However, another unexpected inhibitory interaction between PD-L1 and B7.1 (with B7.1 acting as an inhibitory receptor on T cells) has been described; this interaction between PD-L1 and B7.1 is blocked by anti-PD-L1 but not by anti-PD-1 antibodies. While the PD-L1: PD-1 interaction is considered the most important mediator of tumor immune resistance, the importance of PD-L2: PD-1 and PD-L1:B7.1 in human cancers has not been well studied and those interactions could play a role in the differences in clinical activities of anti-PD-1 and anti-PD-L1 antibodies. It is also possible that these additional interactions may be important in organ specific immune modulation, which could impact relative immune toxicities of these two classes of antibodies.

A multicenter phase I trial of BMS-936559 has been done on 207 patients including 75 NSCLC, 55 melanoma, 18 colorectal cancer, 17 renal cell cancer, 17 ovarian cancer, 14 pancreatic cancer, 7 gastric cancer, and 4 breast cancer cases [52]. This study reported that the drug was well tolerated with durable tumor regression in 6-17% and prolonged stabilization of disease [52].

Biomarkers for Immunotherapies

Research on predictive biomarker for lung cancer immunotherapy is at a relatively early stage and there are a number of potentially important issues. One may question whether a predictive biomarker/companion diagnostic is even necessary or possible, given the complexity and dynamic nature of balances between co-stimulatory and co-inhibitory signals and the interactions with their ligands. The biologic significance of tissue micro-localization of biomarker expression remains to be elucidated. More studies are also needed to evaluate the optimal detection method(s) for biomarkers, especially PD-L1; currently, there are several anti-PD-L1 antibodies for immunohistochemistry, none of which has been proven to be widely applicable in routine clinical practice to date. The best time of biomarker testing (e.g. time at diagnosis vs. time at relapse) and the tissue type to be tested (primary vs. metastatic tumor) need to be determined as well. The impact of chemotherapy, molecular targeted therapy or radiotherapy on biomarker expression is another area of further investigation. Finally, evaluation of tumor response to immunotherapeutic agents can be challenging, in that there may be a pseudo progression due to peritumoral lymphocyte infiltration caused by immunotherapeutic agents if conventional imaging study is used to determine the size of tumor as a marker of responsiveness to therapy.

Tumor PD-L1 protein expression may predict response to drugs targeting PD-1 pathway such as PD-1 inhibitor Nivolumab. However, detection of PD-L1 protein is limited by the lack of standardized immunohistochemical methods and variable performance of antibodies. As an alternative to immunohistochemistry, in situ tumor PD-L1 mRNA expression has been used and shown to be associated with increased TILs and better outcome in breast carcinomas [53]. The purpose of this study was to correlate the PD-L1 mRNA expression with clinical variables in primary breast carcinomas. The fluorescent RNAscope paired-primer assay was used to quantify in situ PD-L1 mRNA levels in 636 stage I-III breast carcinomas on two sets of tissue microarrays (TMAs). TILs were assessed by HE stain and quantitative fluorescence. On both TMAs, 55.7% and 59.5% of cases showed PD-L1 mRNA expression. Higher PD-L1 mRNA expression was significantly associated with increased TILs. Elevated TILs occurred in 16.5% and 14.8% TMAs and were associated with estrogen receptor negative status. In this study, PD-L1 mRNA expression was associated with longer recurrence-free survival which remained significant in multivariate analysis including age, tumor size, histologic grade, nodal metastasis, hormone receptor, HER2 status, and the extent of TILs.

One notable recent study reported that the tumor PD-L1 expression reflects an immune-active microenvironment and is the single factor most closely correlated with response to anti-PD-1 blockade [51]. Taube et al. explored PD-1, PD-L1, and PD-L2 expression by tumor cells and infiltrating immune cell subsets, and their relationships to each other and to clinical response to PD-1 inhibitor Nivolumab. In this study, they included 41 patients with NSCLC, renal cell carcinoma, colorectal carcinoma or castration-resistant prostate cancer who were treated in the setting of an early phase trial of Nivolumab at one institution and had evaluable pre-treatment tumor specimens. Significant associations were found among tumor cell PD-L1 expression, the presence of intratumoral immune cell infiltrates, and the PD-1 receptor expression by TILs. Among these parameters, they found that the tumor cell PD-L1 expression was most closely associated with response to ant-PD-1 therapy. PD-L1 expression was also significantly associated with tumor types responding to anti-PD-1 including melanoma and carcinomas of the lung and kidney. Based on this result, they purported that tumor cell PD-L1 expression may be used as the biomarker to identify additional tumor types which may respond to PD-1 pathway blockade. Tumor cell PD-L1 expression correlated with objective response to anti-PD-1 therapy, when analyzing either the specimen obtained closest to therapy or the highest scoring sample among multiple biopsies from individual patients. These correlations were stronger than the associations of PD-1 expression or intratumoral immune cell infiltrates with the response to anti-PD-1 therapy.

Acknowledgment

Author sincerely thanks Dr. Thomas V. Colby at Mayo Clinic Arizona for his invaluable input and insightful discussion on this manuscript.

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