



A narrative overview of the prognostic significance of the immune cellular milieu in tumor draining lymph nodes in non-small cell lung cancer

Praveen Sridhar¹, Anne Sailer²

¹Division of Thoracic Surgery, Department of Surgery, Boston University School of Medicine, Boston Medical Center; Boston, MA, USA;

²Department of Radiology, Yale University School of Medicine, New Haven, CT, USA

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Correspondence to: Praveen Sridhar, MD. Boston University School of Medicine, Surgical Education Office, 88 E. Newton Street, Collamore Building Suite C-515, Boston, MA 02118, USA. Email: praveen.sridhar@bmc.org.

Abstract: Lung cancer is both the most common malignancy in the United States and the most fatal. Non-small cell lung cancer accounts for most lung malignancies and involves a complex immune response that has become the recent target of systemic therapy. Late-stage disease is now effectively treated with immunotherapy. As the role of immunotherapy expands, it is becoming increasingly important to understand the biology of the immune response in patients with non-small cell lung cancer and the association of this response with response to therapy and outcomes. In this narrative overview, we review the role of immune cells comprising tumor draining lymph nodes, the structure of tumor draining lymph nodes, and their role in the tumor microenvironment. To identify relevant immune cell interactions within the tumor draining lymph nodes, we reviewed published papers focusing on tumor draining lymph nodes and delineated the significance of the immune cells to the tumor microenvironment in both animal models and humans. This is the first comprehensive review of tumor-draining lymph nodes and their role in the tumor microenvironment and provides a foundation for further investigating the tumor microenvironment and the role of humoral and innate immune response mechanisms in non-small cell lung cancer.

Keywords: Tumor draining lymph node; non-small cell lung cancer (NSCLC); immune response

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Introduction

There will be an estimated 230,000 new cases of lung cancer by the end of the year 2020, and it remains the leading cause of cancer death among men and women in the United States (1). Non-small cell lung cancer (NSCLC) has a high mutational burden that contributes to host anti-tumor T cell responses through tumor-specific neoantigens. As our understanding of the immunosuppressive mechanisms that allow tumor cells to evade adaptive immunity improve, immune targets for therapy such as the program death protein and ligand (PD-1/PD-L1) and the cytotoxic T

lymphocyte-associated antigen-4 (CTLA-4) have emerged (2,3). Therapeutic targets within the immune system are limited, however, as approximately 80% of patients treated with current immune checkpoint inhibitors (ICIs) will not respond (2). Central to the tumor immune response is the activation of T cells within lymphoid structures and regional lymph nodes.

Tumor-draining lymph nodes (TDLNs) are lymphoid structures that are largely the first organs of metastases in many cancers, where the presentation of neoantigens leads to the activation of lymphocytes (4). The characteristics

of tumor cells and neoantigens in lymph nodes provides one of the first insights into the likely efficacy of ICI. Additionally, TDLNs are important prognosticators in several malignancies, as approximately 30–40% of patients with lung, colorectal, and breast cancer are diagnosed at the time of nodal metastasis (5). It is critical to understand the structure and function of the cellular components of TDLNs which may provide information regarding prognosis and utility of ICI therapy.

Several populations of immune cells in the primary tumor, tumor microenvironment, and regional lymph nodes contribute to the complete immune response to NSCLC. Several studies have attempted to associate patterns of immune cellular clonality, proliferation and protein expression with disease progression and overall survival (2,6-13). Furthermore, these immune responses have been shown to be unique in the TDLN relative to the non-TDLN in several malignancies (14,15). Here we review the immune cellular response in NSCLC within TDLNs and its impact on both prognosis and therapy. We present the following article in accordance with the Narrative Review reporting checklist (available at <https://amj.amegroups.com/article/view/10.21037/amj-20-171/rc>).

Methodology

A literature search was performed using the MEDLINE database using a combination of keywords including “Non small cell lung cancer”, “tumor draining lymph node”, “immune response”, “T-cell”, “regulatory T-cell”, “cytotoxic T-cell”, “dendritic cell”, “natural killer cell”, “tumor associated macrophages”, “antigen presenting cell”, “B-cell”, “humoral immunity”, “innate immunity”, “immune cytoarchitecture”, and “prognosis”. Full text articles written in the English language and published between the years 2000 and 2020 were subjectively assessed by the authors for content and relevance.

Clinical significance of the tumor draining lymph node

Patterns of lymph node drainage in NSCLC

Current prognostication in NSCLC is predicated upon the American Joint Committee on Cancer (AJCC) Tumor, Lymph Node, and Metastasis (TNM) staging system (4). The pattern of lymphatic spread in NSCLC has been described, though increasing scrutiny suggests that our initial understanding of this spread is limited

(16,17). The theorized route of lymphatic spread occurs via intraparenchymal lymphatic drainage to the hilum and mediastinum (17,18). Variations to this traditionally described drainage have been observed, including both skip metastases and micrometastases (17,19). The impact of variable lymphatic drainage on prognosis is not well understood and raises questions regarding our fundamental knowledge of the interaction between primary tumor cells and reactive immune cells, and the biological and clinical significance of this interaction. The role of tumor evasion of secondary lymphoid functions beginning with the TDLN, such as immune cell homing, priming, and anti-tumor reactivity is not fully elucidated (20). NSCLC is one of a few cancers in which immune system evasion has been, to a degree, successfully targeted with ICI. The mechanism by which ICIs exert their influence to evade immune reactivity within the TDLN is the subject of ongoing research.

Microenvironment, cellular composition, and immune markers of the tumor draining lymph node

Lymph node microenvironment as well as the cellular composition may provide insight into the immune response and thereby the prognosis of NSCLC as previous reports have shown immunologic parameters, such as the density of tumor infiltrating lymphocytes, better predict clinical outcomes than TMN staging (13,21-25). Lymphatic flow begins with afferent lymphatic vessels draining into subcapsular sinuses on the convexity of the node and into cortical sinuses. These sinuses course between lymphatic nodules with B-cell germinal centers and progress from the outer cortex to the deeper cortex and finally the medulla of the lymph node before exiting the efferent lymphatics (26). Simply, antigen presenting cells (APCs) induce and stimulate both CD8⁺ (cytotoxic) and CD4⁺ (helper) T cells in the cortical spaces as well as humoral immunity from B-cell stimulation within the cortex and germinal centers.

Paramount to the antitumor response in TDLN in NSCLC are mature dendritic cell (DC) stimulation of CD8⁺ T cells (27). The relative stimulation of cytotoxic T cells to helper T cells has been studied by several investigators as a critical event in the antitumor response (9,27-29). Ito *et al.* demonstrated that the cytokine transforming growth factor beta-1 (TGF-β1) released by tumor cells results in DC apoptosis in TDLNs (27). Additional data in both NSCLC and other cancers suggests that the suppression of CD8⁺ cytotoxic T cells occurs via multiple mechanisms in NSCLC tumor microenvironment (28,29). Immunosuppressive

tumor derived cytokines prevent DC maturation and lead to a tumor supportive milieu via lymphangiogenesis, blood vessel remodeling, and altered immune cell composition in cervical cancer (30). The importance of mature DCs to the anti-tumor response is not limited to the primary tumor microenvironment and TDLN. Tertiary lymphoid structures (TLS) in non-tumor bronchial associated lymphoid tissue with high mature DC density have also demonstrated favorable prognoses in NSCLC (31).

Tumor associated macrophages, or CD169⁺ macrophages have been examined in several animal models and some human solid tumors where they are associated with increase antitumor activity (32-34). Furthermore, CD169⁺ macrophage density correlated with CD8⁺ T cell density, and was in turn associated with fewer cases of lymph node metastases (32). While mature DC and cytotoxic T cell activation are associated with a more robust anti-tumor response and even improved prognoses, several authors have shown that their suppression occurs in conjunction with an upregulation of CD4⁺/CD25⁺ T cells (10,15). This immunosuppressive phenotype results in anergy and immune system evasion.

Cytotoxic T-lymphocyte associated protein-4 (CTLA-4) and programmed death protein-1 and its ligand (PD-1/PD-L1) have both been implicated in the immune evasion of tumors in TDLNs (9,12,15,30,35-37). The expression of PD-L1 on tumor promoting macrophages as well as the increased presence of PD-1 in TDLNs compared to peripheral blood and non-TDLNs has been shown by multiple investigators (9,15,30,36). Consequently, these proteins have increasingly been targeted for therapeutic intervention with ICI in patients with NSCLC. ICI has now improved survival stage IV NSCLC; however, ICI is not indicated in 70–80% of these late-stage patients based on their expression of PD-L1. We will next review the impact of specific immune cell populations within TDLNs in relation to prognosis and therapy.

Key cells involved in the immune response in tumor draining lymph nodes

T cell overview

In patients with early-stage lung cancer, T cells comprise nearly half of all CD45⁺ cells (24). CD4⁺ T cells made 26% of the T cell population while cytotoxic CD8⁺ T cells were responsible for 22% (24). Naive T cells are exposed to tumor neoantigens in lymphoid structures, thereby

establishing an initial immune response. T cell migration in the TDLN occurs via high endothelial venules (HEV), which allow naïve T cells migrate into the paracortex for their initial encounter with antigen presenting DCs. This pattern of migration is necessary to activate the antitumor response (38). T cell activation and function within the TDLN is not only vital to the initial antitumor response but also to immune exhaustion and evasion through peripheral tolerance (6,7,10,12,15,39-44). T cell presence in tumors has been positively correlated with their presence in TDLNs and with survival in NSCLC, though the mechanism of this is not clear (8,11,45-54). We will provide a narrative review of the role of several T cell subtypes within the TDLN and the prognostic significance that these cellular subtypes may have in NSCLC.

CD4⁺ T cells

CD4⁺ T cells participate anti-tumor activity by priming CD8⁺ T cell, recruiting innate immune cells in the tumor bed, and directly promoting tumor cell death. CD4⁺ T cells recognize neoantigens presented on major histocompatibility (MHC) class II molecules expressed by APCs. CD4⁺ T cells may subsequently engage in antitumor CD8⁺ T cell priming or in pro-tumor regulatory T cell (Treg) generation. In the TDLN, neoantigen presentation to naïve CD4⁺ T cells results in the maturation of T-helper (Th) cells (10). Th cells that mature into T helper type 2 cells are then involved in generating the humoral immune response via B-cell activation. Priming of the humoral immune response has been shown to facilitate carcinogenesis and tumor progression in cancer models (55). The Th1 lineage of CD4⁺ T cells, however, is thought to be involved in the antitumor cellular response, as the number of Th1 cells has been shown to correlate inversely with primary tumor size in patients with resectable NSCLC (2,26,55). There may be a prognostic significance of a predominant Th1 presence in the TDLN after treatment with monoclonal antibodies (anti PD-1, anti-CTLA-4, anti-CD137, against TNF alpha receptor 9, anti-CD19), as *in vitro* and murine models of lung carcinoma and melanoma show long-term tumor free remission. Failure to maintain the Th1 response after mAb administration coincides with recurrence with a Th2 predominant response (55).

In a lung adenocarcinoma mouse model, tumor antigen specific CD4⁺ T cells that are activated and proliferate within the TDLN cannot be efficiently primed, rather they become anergic peripheral Treg precursor cells (10). Data

suggests that the same anergic genetic expression signature additionally arises from chronically stimulated CD4⁺ T cells in patients with untreated metastatic melanoma and NSCLC relative to healthy patients. These studies may signify the importance of CD4⁺ T cells in the mechanism of cancer immune evasion and tolerance (10). Specific CD4⁺ T cell subsets are present in different numbers in peripheral blood, lung, and tumor draining lymph nodes (56). In thoracic lymph nodes sampled via endobronchial ultrasound and needle aspirate in human NSCLC patients, the relative depletion of effector CD4⁺ in TDLNs compared to non-draining nodes has been associated with higher tumor PD-L1 expression (12). This may have implications on the selection of patients for PD-1/PD-L1 blockade. CD4⁺ T cells additionally play an important role in the initiation of the antitumor response not only through proliferation of the Th1 lineage but via priming of CD8⁺ T cells.

CD8⁺ T cells

Many studies have characterized the importance of tumor infiltrating CD8⁺ T cells and the initial response to neoantigens (3,8,45-54,57-59). To summarize, neoantigens are presented directly to T cells and are presented via APCs (45). T cell clonal expansion is associated with favorable outcomes in multiple cancers (3). Prior studies have demonstrated an association between the density of CD8⁺ cytotoxic effector T cells at the site of the primary tumor and survival of patients across multiple cancer models (48,57). Despite their association with improved survival and infiltration of tumor nests, antitumor cytotoxic effector T cells are not clearly functionally active within these sites. Functional studies measuring IFN- γ expression as a surrogate for CD8⁺ T cell activity localized CD8⁺ T cell activity to the peritumoral, rather than intratumoral, region. This suggests that while cytotoxic effector T cells infiltrate tumor nests, intratumoral CD8⁺ T cell do not mount a robust antitumor response (13). It is hypothesized that the chronic inflammatory stimulation from tumor cells may also contribute to T cell anergy (46).

The presence of a CD8⁺ T cell response within TDLNs in NSCLC murine models may occur prior to any clinical or radiographic manifestation of disease spread (8). In murine melanoma models, CD8⁺ T cell stimulation within TDLN from lymph nodes by APCs result in a more robust anti-tumoral cytotoxic response than direct stimulation from tumor cells, though the cytotoxic response even from TDLNs was muted in tumors that had been present for longer periods of time (50). Tumors that have been

established for relatively long periods of time may still have high cytotoxic T cell stimulation within TDLNs, however these T cells do not complete maturation to become effective antitumor effector T cells (50). The same data did suggest that re-priming of the T cell response in TDLNs with APCs can re-invigorate T cell stimulation and maturation (50). Subsequent work has revealed the importance of CD103⁺CD8⁺ cells, or resident memory cells to the process of re-priming (49). Analysis of The Cancer Genome Atlas (TCGA) lung cancer dataset revealed that a relatively high expression of CD103⁺ on CD8⁺ cells (T resident memory) conferred a survival benefit (49).

Lastly, high PD-L1 expression in NSCLC provides immune evasion by circumventing T cell activation. Patients with NSCLC with a high level of tumor infiltrating CD8⁺ cells and similar expression patterns of PD-L1 in tumors and their corresponding draining lymph nodes may experience a better overall survival than patients who display variability in PD-L1 expression between tumors and their corresponding metastatic lymph nodes (*Table 1*) (59).

Treg

Tregs are CD25⁺CD4⁺ that express the forkhead/winged helix transcription factor (FOXP3) have been found to suppress the host immune response and present a pathway to immune evasion by inhibiting the activation and maturation of T cells (6-8,10,12,15,39,40,42,43).

Peripheral tolerance of tumor activity occurs via Treg induced anergy. This has been characterized the maturation and differentiation of precursor Treg cells from the CD4⁺ population. Alonso *et al.* demonstrated in murine models that anergic peripheral Treg are present in the TDLN cells. In contrast to studies that demonstrate associations between survival and tumor infiltrating CD8⁺ and CD4⁺ cells, there are a few studies that have demonstrated a concordant prognostic significance of Treg cells in the tumor draining lymph nodes (6-8,10,12,15,39,40,42,43).

In patients with stage I, II, and III NSCLC, a higher proportion of Tregs found within the TDLN was associated with decreased 5-year survival (6). Importantly, the 5-year survival was significantly lower in patients with stage I disease as well (6). Patients with high proportion of Treg in TDLN had 63.5% 5-year survival compared to 84.4% for low Treg TDLN tumors (P=0.0056). Resected stage I NSCLC had 91.4% 5-year survival in Treg-low group compared to 72.1% in Treg-high group (P=0.0147) (6). The relative expression of FOXP3 in TDLNs in stage I patients appears to influence survival. Hanagiri *et al.* demonstrated that a high

Table 1 Summary of studies with notable findings pertaining to the prognostic significance of tumor draining lymph nodes in NSCLC

Author, journal	Stage	N	Cell type	Summary of findings
Schneider <i>et al.</i> , <i>Journal of Thoracic Oncology</i> 2011	I-III	20	Treg	Tregs enhanced in TDLN for adenocarcinoma but not squamous cell carcinoma
Hanagiri <i>et al.</i> , <i>Lung Cancer</i> 2013	I-III	158	Treg	Patients with high proportion of Treg in TDLN had 63.5% 5-year survival compared to 84.4% for low Treg TDLN tumors (P=0.0056) Resected Stage I NSCLC had 91.4% 5-year survival in Treg-low group compared to 72.1% in Treg-high group (P=0.0147)
Hanagiri <i>et al.</i> , <i>Anticancer Research</i> 2014	I	131	Treg	Evaluation of TDLNs for FOXP3 expression 5-year survival for low FOXP3 group 90.3% compared to 79.3% in high FOXP3 expression group (P=0.0419)
Reineke <i>et al.</i> , <i>EJR Open Research</i> 2017	II-IV	11	Treg	Evaluate T cell composition and PD-1 expression in TDLN High Treg frequency in TDLN of 1 patient with rapid mortality suggesting immune evasion
Yang <i>et al.</i> , <i>Cancer Medicine</i> 2018	I-IV	58	CD8 ⁺ T cell	Inconsistent PD-L1 expression between primary tumors and TDLNs in nearly 40% of patients For 60% of patients with consistent expression of PD-L1 in tumor and TDLN, CD8 ⁺ TIL correlated with longer overall survival
Murthy <i>et al.</i> , <i>Lung Cancer</i> 2019	I-IV	20	Treg	12 TDLNs and non-draining lymph node aspirates via endobronchial ultrasound for evaluation of immunophenotype Lower density of CD4 ⁺ T cells in TDLN compared to nondraining nodes Higher Tregs in TDLN suggesting immunosuppressive phenotype in TDLNs

NSCLC, non-small cell lung cancer; TDLN, tumor-draining lymph node.

relative FOXP3 expression in TDLNs was associated with a significantly worse 5-year survival (7). Specifically, 5-year survival for low FOXP3 group 90.3% compared to 79.3% in high FOXP3 expression group (P=0.0419) (7). Prior studies have shown conflicting associations between survival and Treg cell volume both generally and in tumor stroma, specifically (39,41,43,44,47). This raises the possibility that Treg expression specifically within the TDLN may carry meaningful potential as a tool for prognostication compared to tumor or systemic Treg expression.

B cells

Stankovic *et al.* found that B cells were the second most prevalent immune cell type in NSCLC tumors, with CD19⁺ B cells comprising 16% of all CD45⁺ immune cells (24). Kargl *et al.* found a 7-fold increase in B cells within the tumor region when compared to normal lung tissue in NSCLC patients (2). The percentage of naïve and plasma B cells has been shown to be similar between tumor and normal peripheral lung tissue; however memory B cells may

be associated with tumor tissue in both adeno and squamous cell carcinoma of the lung (24). B cells are activated in secondary lymphoid organs in the primary follicle following antigen binding, and proliferate and expand to form secondary follicles that progress to germinal centers after stimulation from a CD4⁺ T effector cells (57).

There is a paucity of literature associating the B cell response within tumor draining lymph nodes and survival, however pooled data suggests that there may be an association between the presence of intra and peritumoral B cell presence and improved survival (43). There is currently no data associating the density or function of B cells within the TDLN and survival. Several studies have associated the presence of B cells within TLS in the region of the tumor with improved survival for NSCLC patients; however, these findings have not been expanded to the TDLN (29,31,43,60).

APCs

APCs are a vital initiator of the antitumor immune

response, as they can activate both CD4⁺ and CD8⁺ effector cells, thereby kickstarting the immune response to cancer (52). APCs consist of tumor-associated macrophages, DCs, and natural killer (NK) cells. While there is data correlating APC intra- and peritumoral density and subtype with prognostic information, these findings have largely not been established in TDLNs (16,27,32,52,61-68). While there is no data to specifically associate survival and APC function or density within the TDLN, there are several studies that provide some insight into the mechanism of immune evasion at the level of antigen presentation and effector cell priming within the TDLN (27,41,65).

DCs

DCs migrate from tumor tissue to regional TDLNs where they stimulate naïve T cells (63). There is no survival association with DC function in TDLN; however, important data regarding immune evasion by tumor cells has been elucidated in TDLN tissue in multiple studies owing to upregulation of PDL family co-inhibitory molecules and inhibitory cytokine release from TILs (63,65). Dieu-Nosjean *et al.* demonstrated an association between mature DC in TLS and overall and disease-free survival in early-stage NSCLC (31).

Tumor-associated macrophages (TAMs)

Macrophages were found to comprise between 5% and 15% of the immune infiltrate in NSCLC (2,24). TAMs differentiate into either an antitumor M1 phenotype or a protumor, immunosuppressive, M2 phenotype. Macrophages have been shown to have both a favorable and an unfavorable prognostic significance in NSCLC in several studies reviewed by Suzuki *et al.* (13). These studies have focused primarily on peri- and intratumoral TAM density and function, rather than within the TDLN.

NK cells

NK cells are part of the innate immune system, play a key role in the host antimicrobial and antiviral response, and can initiate an anti-tumor response without the requisite tumor associated antigen (26). In both murine models and human lungs, NK cells are more differentiated relative to circulating NK cells with a lesser degree of effector function. Pooled data suggests that a relatively high intratumoral and peritumoral NK cell activity correlates to improved overall and disease-free survival (43). Similar to other APCs, there is no data to suggest that TDLN NK density or function has a similar association.

Conclusions and future directions

The importance of the immune cell milieu within the tumor and peritumor microenvironment has been studied extensively. Similar research regarding immune function and cytoarchitecture with respect to the TDLN is underway. Results from these studies have provided new insight regarding the prognostic significance of the immune cell composition and function within TDLNs as well as the potential for ICIs. While the focus of this research has been effector T cells, the lack of data with respect to the prognostic impact of APCs and B cells within TDLNs provides a new potential for future investigation.

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