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Tumor-draining lymph nodes demonstrate a suppressive immunophenotype in patients with non-small cell lung cancer assessed by endobronchial ultrasound-guided transbronchial needle aspiration: A pilot study

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ABSTRACT

Objectives: Tumor draining lymph nodes (TDLN) are key sites of early immunoediting in patients with non-small cell lung cancer (NSCLC) and play an important role in generating anti-tumor immunity. Immune suppression in the tumor microenvironment has prognostic implications and may predict therapeutic response. T cell composition of draining lymph nodes may reflect an immunophenotype with similar prognostic potential which could be measured during standard-of-care bronchoscopic assessment. In this study, we compared the immunophenotype from different sites within individuals to primary tumor characteristics in patients with NSCLC to see whether there were tumor-regional differences in immunophenotype which could be evaluated from transbronchial needle aspirates.

Materials and Methods: Twenty patients were enrolled in this study and had tissue (lymph node aspirates and/or peripheral blood) obtained during standard of care bronchoscopy with endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) for diagnosis or staging of known or suspected NSCLC. Aspirates and blood underwent flow-assisted cell sorting and a subset of sorted effector T cells underwent RNA quantitation to determine feasibility of this approach. Immunophenotypic patterns from twelve patients with paired data from tumor-draining and non-tumor draining lymph nodes (NDLN) were compared relative to one another and based on PD-L1 immunohistochemistry and primary tumor histology.

Results: TDLN had significantly fewer $CD4^+$ T cells (12.68% vs 27%, $p = 0.002$) and significantly more regulatory T cells (Treg, 12.03% vs 9.52%, $p = 0.03$) relative to paired NDLN suggesting tumor-regional immunosuppression. There were significantly more Treg in NDLN relative to paired PBMC (9.52% vs 5.6%, $p = 0.016$). Patients with PD-L1 expression $\geq 50\%$ had significantly greater tumor-regional $CD4^+$ T cell depletion compared to patients with PD-L1 expression $< 50\%$ (-35.98% vs -1.89%, $p = 0.0357$; negative values represent absolute difference between paired TDLN and NDLN).

Conclusions: In patients with NSCLC, TDLN have a suppressive immunophenotype correlating with tumor PD-L1 status and can be assessed during routine EBUS-TBNA.

1. Introduction

There is increasing awareness of the role of the tumor-draining lymph node (TDLN) as an early site of both immune activation and evasion by non-small cell lung cancers (NSCLC) [1,2]. While there have been tremendous advances in our understanding of the dynamic process of immunoediting in the tumor microenvironment and its prognostic significance, less is known about changes in effector and regulatory mechanisms at the level of the TDLN [3–7]. There are several features of both the tumor and TDLN that attenuate effective immune activation, including defects in antigen presentation, induction of anergy in effector T cells, an increase in regulatory T cells (Treg) and increased

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expression of tolerogenic cytokines 2,4,8,9].

Tumor infiltration by Tregs and M2-polarized tumor-associated macrophages is associated with worse survival and inferior response to chemotherapy in NSCLC [9,10]. The dense Treg infiltration observed in primary tumors has also been reported in resected tumor-infiltrated lymph nodes, and the proximity of Tregs to tumor cells on immunohistochemistry has been demonstrated to be an independent predictor of poorer survival [11,12]. Depletion of Tregs observed in the course of neoadjuvant chemotherapy has been found to correlate with improved clinical response [13]. This growing body of evidence suggests that Treg-mediated immunosuppression in the tumor microenvironment may also be evident in the draining lymph node and may have prognostic implications in the setting of NSCLC, however it remains to be determined whether this can reliably be assessed in TDLN [2,6,[9,14].

In addition to changes in T cell subpopulations, expression of counter-regulatory surface molecules (immune checkpoint proteins) is increased in tumor-infiltrating lymphocytes and tumor cells of patients with NSCLC [15]. These checkpoint proteins block co-stimulation of naïve T cells and promote Treg proliferation, inducing anergy towards tumor cells enabling disease progression. Programmed cell death receptor protein ligand-1 (PD-L1) is a checkpoint protein expressed on tumor cells which has emerged as an important therapeutic target and as a biomarker predicting response to the anti-PD-L1 monoclonal antibody pembrolizumab in NSCLC [16]. Tumor PD-L1 expression has been independently associated with younger age at presentation, higher tumor grade and pathologic stage and may be associated with better overall survival in defined subgroups of patients [17,18]. However, measurement of PD-L1 by immunohistochemistry has demonstrated heterogeneity even in paired samples obtained from the same tumor, suggesting a role for a more consistent biomarker to predict response to therapy [19,20].

Bronchoscopy with endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) has become the first-line approach to mediastinal staging assessment [21,22]. EBUS-TBNA has been employed to characterize the immune composition of lymph nodes in patients with NSCLC. However the optimal comparator, whether to paired peripheral blood, paired lymph nodes from individual subjects or lymph node aspirates from non-involved subjects, has not been established [23,24]. EBUS-TBNA bronchoscopy may provide an opportunity to generate immunologic data with prognostic significance as part of the routine staging evaluation.

Establishing the immunophenotype of thoracic lymph nodes in patients with NSCLC is a foundation for exploring the diagnostic, prognostic and therapeutic potential of the nodal microenvironment. By comparing transbronchial needle aspirates from TDLN and non-tumor draining lymph nodes (NDLN) within individual subjects we aim to establish whether there are regional differences in immunophenotype which can be measured during standard-of-care EBUS-TBNA, and which could have prognostic significance.

2. Materials and methods

2.1. Patient Selection and Sample Acquisition

Patients with known or suspected NSCLC undergoing routine standard of care endobronchial ultrasound (EBUS) bronchoscopic lymph node sampling for diagnosis, staging and tissue acquisition were recruited for participation in this study at New York University Medical Center and Bellevue Hospital Center in New York between August 2016 and December 2017.

EBUS-TBNA was performed with an Olympus 21- or 22-gauge needle (Olympus Corporation, Tokyo, Japan) at the discretion of the pulmonologist performing the bronchoscopy. All bronchoscopies were performed under general anesthesia administered by an anesthesiologist. After clinical tissue acquisition at each site, one additional aspirate was collected in normal saline for study purposes from a tumor-draining lymph node and, when available, a non-tumor draining lymph node. NDLN were defined as N2 or N3 nodes (as per the 7th edition, International Association for the Study of Lung Cancer guidelines) which were > 5 mm and < 10 mm in short axis and did not demonstrate significantly increased uptake of 18F-fluorodeoxyglucose (FDG) on positron emission tomography (PET). TDLN were defined as N1 or N2 nodes, enlarged ≥ 10 mm in the short axis and/or had increased FDG-uptake on PET. Peripheral blood (10 mL) was obtained from all subjects at the time of bronchoscopy. PD-L1 expression on tumor cells isolated from either primary tumor or metastatic sites was performed as per standard of care by the NYU pathology laboratory when clinically indicated using the anti-PD-L1 22C3 pharm Dx immunohistochemical assay (Dako, Inc. Carpinteria, California, United States of America), and when performed the result (quantitated from 0 to 100%) was noted for analysis. This protocol received Institutional review board (IRB) approval for human subjects research from the New York University School of Medicine IRB (Protocol #R8896) and informed consent was obtained and documented from all participants prior to bronchoscopy.

2.2. Flow Cytometry

Lymph node aspirates were made into single cell suspension by gentle disaggregation through a 70 μm cell strainer and washed with phosphate buffered saline (PBS). Cell suspensions were centrifuged and washed with red blood cell lysis buffer (BD Biosciences, Franklin Lakes, New Jersey, United States of America), centrifuged and washed again with PBS. Peripheral blood underwent Ficoll-Paque separation, after which the buffy coat was aspirated and washed with PBS twice prior to staining. Cell staining was performed with the following fluorophore-conjugated anti-human monoclonal antibodies: CD4-FITC, CD8-BV450, CD25-PECy7, CD127-APCR700 and CD45RO-PECF594 (BD Biosciences) which were incubated for 20 min at 4^0C with the sample, then centrifuged and re-suspended in 100 μL PBS. Given the preliminary nature of this study, a narrow panel was designed for flow cytometry in order to provide broad characterization of the immune contexture of mediastinal and hilar lymph nodes. Flow assisted cell sorting (FACS) was performed using the BD FACSAria II flow cytometer (BD Biosciences) which was used to count and sort cells into three populations: $CD8^+$ (cytotoxic T cells), $CD4^+CD25^-$ (effector $CD4^+$ T cells) and $CD4^+CD25^+CD127^{low}$ (Treg). The transcription factor FOXP3 expression is commonly used to help identify Treg, and the population of $FOXP3^+$ T cells has been identified as a clinically significant subset of tumor-infiltrating T cells which enable evasion of immune surveillance. However, as an intracellular stain its use precludes subsequent RNA analysis [25]. FOXP3 expression has been found to be strongly correlated with $CD127^{low}$ expression on T cells, with $CD4^+CD25^+CD127^{low}$ consistently associated with a suppressive phenotype consistent with Treg [25,26]. All FACS analyses were performed in FlowJo v10 software (FlowJo, LLC, Ashland, Oregon, United States of America) with $CD4^+$ and $CD8^+$ T cells reported as a function of live cells and Tregs as a function of $CD4^+$ T cells.

2.3. RNA analysis

Once sorted, the $CD8^+$ and $CD4^+CD25^-$ cells (non-Treg cells) were processed for RNA extraction and mRNA quantitation. Sorted specimens from TDLN, NDLN and peripheral blood were processed for extraction of RNA and frozen at -80 °C within 30 min of sorting. Effector T cell populations (including $CD8^+$ and $CD4^+CD25^-$ sorted cells) were combined and RNA was extracted using the Qiagen RNeasy™ kit (Qiagen, Hilden, Germany), following the manufacturer's protocol, and RNA quality was measured using the Nanodrop™ spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, United States of America) with an appropriate 260/280 ratio for all samples analyzed. Extracted RNA was then processed using the NanoString™ system

(NanoString Technologies, Inc, Seattle, Washington, United States of America) with hybridization to the nCounter® PanCancer OncoImmune panel as per the manufacturer's protocol [27].

2.4. Statistical analysis

T cell populations in paired samples from TDLN, NDLN and peripheral blood were compared by two-tailed Wilcoxon matched-pairs signed-rank tests. The absolute difference between proportions (rather than number of cells counted) of T cell populations of interest in an individual's TDLN and NDLN (ΔCD4) was used to reflect tumor-regional immunophenotype (with the NDLN serving as the control or "expected" for each population of interest in an individual) and this value was used along with tumor cell surface PD-L1 expression to generate a simple linear regression model. Negative values of ΔCD4 were representative of a higher proportion of $CD4^+$ T cells in the NDLN compared to TDLN and positive values represent a higher proportion in the TDLN compared to paired NDLN. Unpaired comparison of T cell populations based on other characteristics was performed with the Mann-Whitney test. Statistical analyses were performed using GraphPad Prism version 7.03 (GraphPad Software, Inc., San Diego, California, United States of America).

3. Results

3.1. Patient enrollment and sample collection

Of 20 subjects enrolled, all had adequate data for PBMC analysis and 12 had adequate samples with complete data from TDLN and NDLN available for analysis (with adequacy defined by a count of $> 0.25 \times 10^6$ cells/μL on Trypan blue viability screen performed prior to FACS). The eight patients excluded from lymph node analysis had samples which were hypocellular ($< 0.25 \times 10^6$ cells/μL on viability screen), were subsequently found to have a benign diagnosis or did not have a NDLN meeting clinical criteria for needle aspiration (e.g. a NDLN < 5 mm in size); however, results from FACS of peripheral blood from these subjects were included in PBMC-related analyses when appropriate. Of patients analyzed, 50% had a pathologic diagnosis of lung adenocarcinoma, 42% had squamous cell lung cancer and 8% had poorly-differentiated non-small cell lung cancer (Table 1).

3.2. Differences in lymph node T cell subpopulations

In paired lymph node samples from individual subjects with NSCLC, there was a significantly lower proportion of $CD4^+$ T cells in TDLN compared to paired NDLN (12.7% vs 27%, p = 0.002, 96.14% CI of median of differences 0.89%–30.72%) (Fig. 1A). This relationship persisted when Tregs were excluded—there were fewer $CD4^+CD25^-CD127^{high}$ T cells (effector $CD4^+$ T cells, "eCD4") in paired samples of TDLN vs NDLN (13.5% vs 29.1%, p = 0.006, 97.85% CI of median of differences 0.13%–39.79%) (Fig. 1B). Parallel to these observations, there were more $CD4^+CD25^+CD127^{low}$ Tregs in TDLN vs NDLN (12% vs 9.5%, p = 0.03, 96.14% CI of median of differences -3.6-0.8%) in paired analysis (Fig. 1C), supporting a pattern of tumor-regional immunosuppression reflected in TDLN T cell composition.

Table 1
Demographic data of patients with complete data from TDLN, NDLN and PBMC. One subject's tumor was a poorly-differentiated non-small cell lung cancer and a more specific histologic subtype could not be identified.

Age, mean *(range)*	64.8 *(56 – 87)*			
Sex, percent *(number)*	Female 50% (6)		Male 50% (6)	
Race/Ethnicity	White 75%	Black 16.7%		Asian 8.3%
Smoking history	Present 91.7%		Absent 8.3%	
Pathologic stage	**I** 33.3%	**II** 8.3%	**III** 41.7%	**IV** 16.7%
Histologic subtype	Adenocarcinoma 50%		Squamous cell 42%	

$CD8^+$ T cells were similar in TDLN and NDLN, with no significant difference in the proportion of $CD8^+$ T cells observed (5.7% vs 6.8%, p = 0.206, 96.14% CI of median of differences -0.48-4.57%). Peripheral blood T cell populations were similar to those found in paired TDLN and NDLN with respect to $CD8^+$ and $CD4^+$ T cells, however there were significantly more Treg in TDLN compared to paired PBMC (12% vs 5.6%, p = 0.003, CI 2.19%–12.35%) and NDLN compared to PBMC (9.5% vs 5.6%, p = 0.016, CI -0.58-8.79%), suggesting tumor-regional Treg expansion and/or recruitment Fig. 2. There was a trend towards a higher proportion of $CD8^+$ T cells in TDLN of subjects with squamous cell lung cancer compared to those with lung adenocarcinoma, although this was not a statistically significant observation (Supplementary Fig. 1).

The implications of tumor metastasis to the TDLN with respect to TDLN T cell composition were investigated within the scope of available data (Supplementary Fig. 2). Of the 12 subjects with complete TDLN-NDLN pairs, 5 had cytopathologic evidence of tumor cells in the TDLN (Table 1). The absolute value of difference between T cell populations of interest between an individual's TDLN and NDLN was used as a measure of tumor-regional variance, and these values were stratified based on presence or absence of tumor in the TDLN. While our sample size was small to detect statistically significant differences, the mean ΔCD4 was lower for subjects with tumor-involved TDLN vs those without (−22.9% vs −7.7%), with a similar difference observed in ΔeCD4 (−26.6% vs 8.3%) and higher mean ΔTreg in tumor-involved TDLN-NDLN pairs than in those without involvement of the TDLN (7.4% vs 2.4%).

3.3. Relationship between lymph node immunophenotype and tumor PD-L1 status

The association between tumor-regional $CD4^+$ T cells and PD-L1 expression on tumor cells, measured as part of standard-of-care immunohistochemical assay, was evaluated. PD-L1 status was classified as "high" (≥50%) or "low" (< 50%) and ΔCD4 was compared between the $PD\text{-}L1^{high}$ and $PD\text{-}L1^{low}$ groups. A negative ΔCD4 value in an individual indicates a lower absolute value in proportion of $CD4^+$ T cells in that individual's TDLN compared to their own NDLN. Amongst patients in the $PD\text{-}L1^{high}$ group, ΔCD4 was significantly lower than in the $PD\text{-}L1^{low}$ group (−36% vs −1.9%, p = 0.036, 96.4% CI 2.58%–67.81%) (Fig. 3), suggesting an association between greater tumor-regional $CD4^+$ T cell depletion (as reflected in the difference between proportion of $CD4^+$ T cells in the TDLN and NDLN) with higher tumor PD-L1 expression. A significant difference was also observed with tumor-regional $eCD4^+$ T cells but not with Treg. The $PD\text{-}L1^{high}$ group had a lower ΔeCD4 compared to the $PD\text{-}L1^{low}$ group (-26% vs -1.4%, p = 0.0357, 96.4% CI 3.8%–62.4%), suggesting tumor-regional depletion of effector $CD4^+$ T cells rather than Treg may contribute more to this observation (Supplementary Fig. 3). This relationship was further characterized through simple linear regression plotting ΔCD4 against tumor PD-L1 expression, with a negative relationship defined by a slope of -0.459 ± 0.173, ($R^2 = 0.5399$, p = 0.038)—higher PD-L1 expression was associated with greater tumor-regional $CD4^+$ T cell depletion (Supplementary Fig. 4).

3.4. Feasibility of evaluating gene expression in sorted cells isolated from EBUS-TBNA specimens

To determine the feasibility of carrying out expression analysis of these T cell samples, mRNA extracted from 3 subjects' paired samples of effector T cells (Teff, comprising $CD8^+$ and $eCD4^+$ T cells) sorted from TDLN, NDLN and PBMC were analyzed using the NanoString™ platform. Absolute RNA counts were normalized for positive controls as per the manufacturer's specifications and the genes with highest expression in Teff cells isolated from TDLN, NDLN and PBMC are summarized in Supplementary Table 2. Results from RNA quantitation were

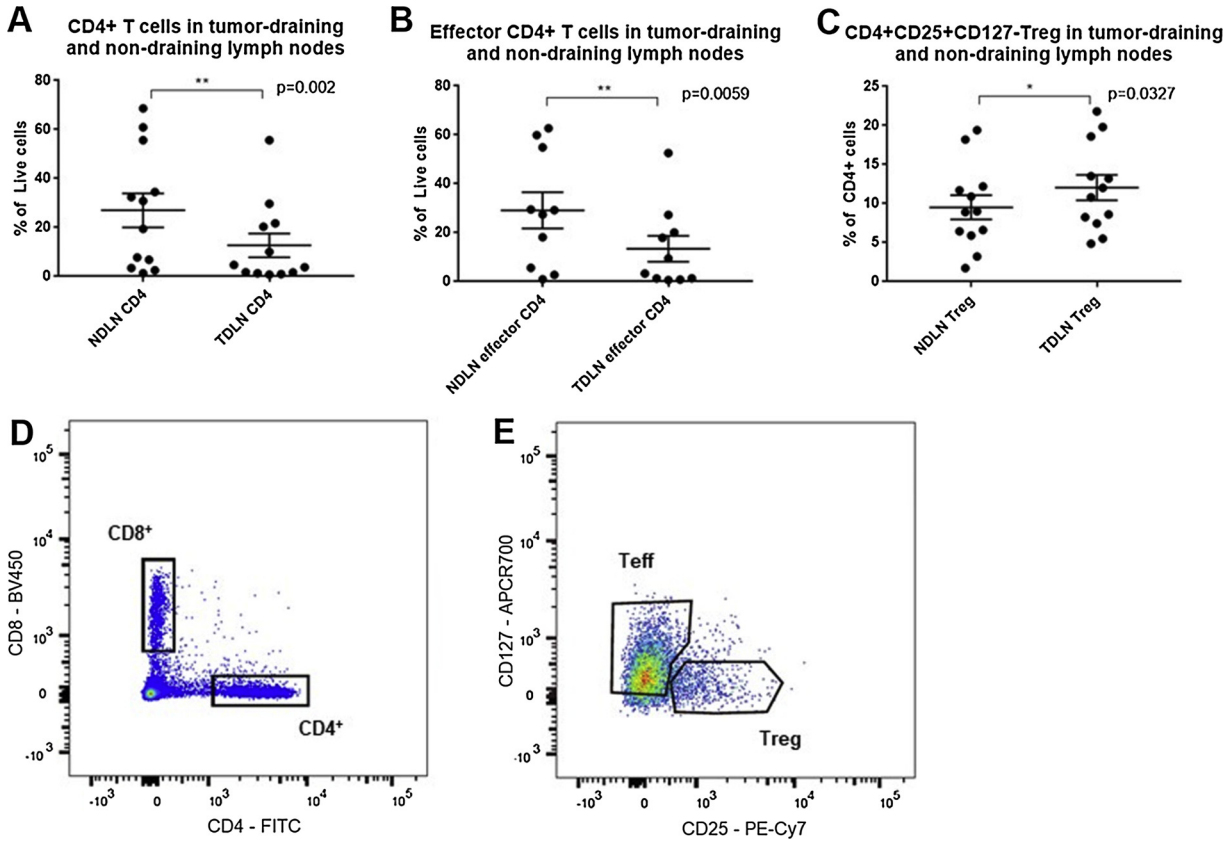

Fig. 1. (A–C) The dot plot show individual patient samples' values (mean and SE) of T cell populations identified by flow cytometry. Paired comparison of means by Wilcoxon matched-pairs signed-rank tests are also shown, with (A) describing differences in proportion of CD4 + T cells in TDLN and NDLN, (B) describing differences in proportion of non-Treg $CD4^+$ T cells and (C) showing a higher proportion of Tregs in TDLN vs paired NDLN. (D) and (E) are representative flow cytometry color density plots depicting $CD8^+$ and $CD4^+$ T cells within a lymphocyte gate (D) and $CD4^+CD25^{low}$ (Teff) and $CD4^+CD25^{high}CD127^{low}$ (Treg) cells within a $CD4^+$ T cell gate (E).

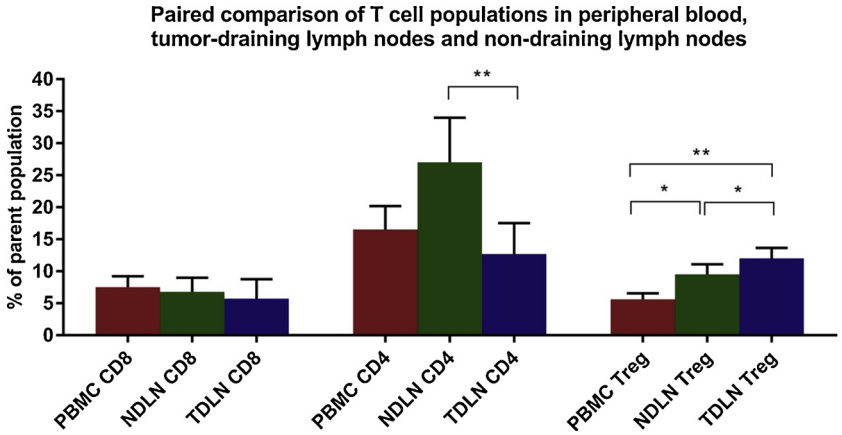

Fig. 2. Mean proportion of T cell subsets by tissue compartment in patients with NSCLC with comparison of means by two-tailed Wilcoxon matched-pairs signed-rank tests. The proportions of CD8 T cells were not significantly different by compartment. There were significantly fewer CD4 + T cells in TDLN compared to paired NDLN 12.7% vs 27%, p = 0.002 CI 0.89%–30.72%). There was a stepwise increase in the proportion of Treg from PBMC to NDLN (5.6% vs 9.5%, p = 0.016, CI -0.58-8.79%), NDLN to TDLN (12% vs 9.5%, p = 0.033, 95% CI -3.6-0.8%) and PBMC to TDLN (5.6% vs 12%, p = 0.003, CI 2.19%–12.35%) suggesting increased tumor-regional immunosuppression evident at the level of thoracic lymph nodes.

normalized to a z-score and plotted on a heat map using unsupervised hierarchical clustering with Euclidean distance measures and average linkage to identify patterns of gene expression [Supplemental Fig. 4]. Given the small sample size, specific conclusions regarding RNA expression cannot be drawn, however the heat map demonstrates broadly consistent patterns of gene expression within Teff from different lymph nodes within individuals. There were, however, differences in expression of genes responsible for T cell co-stimulation between TDLN and NDLN within individual subjects in a pattern of expression suggestive of exhaustion in Teff cells isolated from the TDLN. Specifically, expression of the costimulatory factor CD28 was lower in TDLN vs NDLN (mean mRNA count of 32.01 vs 65.17) as was expression of inducible T cell costimulator (ICOS) also known as CD287 (mean mRNA count of 66.38 vs 102.59), though the small sample size precludes assessment of statistical significance of this observation [Supplementary Table 2].

4. Discussion

NSCLC is now recognized to engage in a dynamic immunologic evolution mediated by tumor, the host and as a consequence of therapeutic interventions [28,29]. This study demonstrates that the TDLN has a distinct suppressive immunophenotype relative to paired NDLN in patients with NSCLC with the TDLN having a lower proportion of effector $CD4^+$ T cells and a higher proportion of Treg than the paired

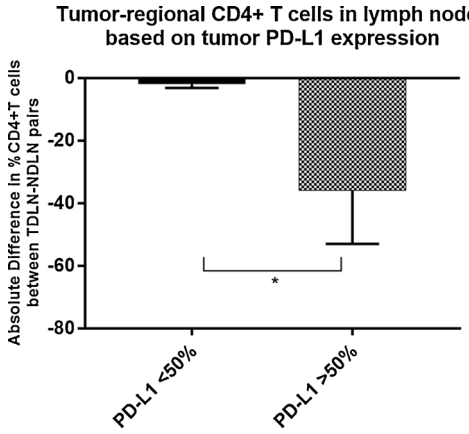

Fig. 3. ΔCD4 (mean and SE) based on PD-L1 status (PD-L1^{low} < 50% vs PD-L1^{high} ≥50%) with significantly lower ΔCD4 in the PD-L1^{high} patients (-36% vs -1.9%, p = 0.036, 96.4% CI 2.58%–67.81%) suggesting greater tumor-regional CD4 + T cell depletion in patients with higher tumor PD-L1 expression.

NDLN. We have also demonstrated an association between CD4$^+$ T cell depletion in the TDLN and PD-L1 status, which may suggest an adjunctive marker to help guide selection of immunotherapies. These findings further support feasibility of using a single needle aspirate obtained by EBUS-TBNA to perform immunophenotyping of mediastinal lymph nodes.

The observation of depletion of CD4$^+$ T cells and increased Tregs in tumor-regional lymph nodes supports findings reported by other investigators on how the tolerogenic state of the tumor microenvironment may be driven by its interaction with the tumor draining lymph node. Van de Ven, et al reported increased frequency of the T cell exhaustion marker PD-1 on CD4$^+$ and CD8$^+$T cells in TDLN of subjects with lung cancer compared to T cells isolated from paired PBMC, with a higher proportion of PD1$^+$CD8$^+$ T cells in the TDLN expressing the proliferation marker Ki67, suggesting induction of tumor-regional anergy [24]. In a small subset of patients' analysis their group suggested there may be fewer PD1$^+$CD4$^+$ and PD1$^+$CD8$^+$ T cells in NDLN versus TDLN in paired analysis, although this was not a statistically significant observation. We did not observe significant differences in regional CD8$^+$ T cells, however it is possible that the magnitude of difference, particularly when stratified by presence or absence of tumor metastasis, may require a larger cohort to fully characterize. Dysfunctional antigen presentation has also been found in the TDLN of subjects with lung cancer, with lower expression of CD80/CD86 by dendritic cells and upregulation of TGF-β and IL-10 reflecting a tolerogenic environment similar to what has been reported in primary tumors [23]. In an elegant mouse model of lung adenocarcinoma by Alonso, et al, the mechanism of immunosuppression extending beyond the tumor to the TDLN was shown to be driven by recruitment of peripherally induced Treg which suppressed robust early tumor-specific CD4$^+$ T cell expansion [30]. This suggests potential diagnostic and prognostic significance may be provided by a more thorough understanding of the state of immune activation or suppression in the TDLN. The association between T cell subpopulations in the TDLN and tumor PD-L1 expression has not to our knowledge been previously explored, however Lizotte, et al, in a detailed immunophenotypic assessment of the NSCLC primary tumor environment demonstrated distinct "hot" and "cold" phenotypes with higher PD-L1 expression associated with more FOXP3$^+$ Treg and PD-1$^+$CD8$^+$ T cells, suggestive of an exhaustion phenotype [3]. This parallels our finding of depletion of tumor-regional CD4$^+$ T cells in subjects with higher tumor PD-L1 expression.

This preliminary feasibility study was designed as a proof of principle with the goal of identifying differences in immunophenotype between lymph nodes within individual subjects using EBUS-TBNA. The number of enrolled subjects was low given the importance of having adequate T cells from clearly defined TDLN and NDLN which was not always feasible. Further investigation will be conducted with more detailed characterization of immune cells employing multiparametric flow cytometry and, given the preliminary observation of significant differences between TDLN and NDLN we have demonstrated, increased emphasis on contemporaneous characterization of the immunophenotype of the TDLN and primary tumors. We also aim to conduct long-term follow-up of subjects enrolled to understand associations between immunophenotype and the natural history of disease as well as possible predictive value of response to chemotherapy and immunotherapy. The preliminary observations from our NanoString™ RNA analysis suggest feasibility of T cell functional assessment of cells sorted from EBUS-TBNA specimens.

Tumor characteristics may be reflected in the immunophenotype of the TDLN and a more detailed characterization may provide biomarkers to guide therapy and develop better models for prognostication and staging. Our findings indicate that cellular and functional immunophenotyping can be performed with specimens acquired during routine bronchoscopic assessment of patients with NSCLC.

Declaration of Competing Interest

The authors have no conflicts of interest to report related to this publication. Please see "Acknowledgments" for a listing of funding sources.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2019.08.008.

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