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Original Article

Infiltration of metastatic lymph nodes with PD-1$^+$ T cells is associated with improved disease-free and overall survival in resected N$^+$ NSCLC

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Abstract: Tumor metastases to regional lymph nodes are associated with worse outcome for patients with resected non-small cell lung cancer (NSCLC), but there is a wide variation in survival. We hypothesized that infiltration of tumor-involved lymph nodes with activated effector T cells would impact subsequent outcome. A total of 54 lymph nodes (27 N$^+$ and 15 N$^-$ collected from 12 patients with Stage IIB ($T_2N_1M_0$) and 12 N$^-$ lymph nodes collected from 10 patients with Stage IIA ($T_2N_0M_0$) who underwent lymphadenectomy during surgical management of their NSCLC) were analyzed for effector T cells expressing activation markers PD-1 and TIM-3 using the *Opal*-multiple immunofluorescence assay. The frequency of CD3$^+$CD8$^+$ (P=0.0001), CD3$^+$CD8$^+$TIM-3$^+$ (P<0.0001), and CD3$^+$CD8$^+$TIM-3$^+$Ki-67$^+$ (P<0.0001) T cells was greater in lymph nodes of IIA patients compared with IIB patients; however the frequency of CD3$^+$CD8$^+$PD-1$^+$ (P=0.0086), CD3$^+$CD8$^+$TIM-3$^+$ (P=0.0129), CD3$^+$CD8$^+$PD-1$^+$Ki-67$^+$ (P<0.0001) and CD3$^+$CD8$^+$TIM-3$^+$Ki-67$^+$ (P=0.0001) T cells was greater among the tumor involved (N$^+$) nodes of N1 patients compared with the tumor-uninvolved (N$^-$) nodes. The frequency of intranodal CD3$^+$CD8$^+$, CD3$^+$CD8$^+$PD-1$^+$ and CD3$^+$CD8$^+$PD-1$^+$Ki-67$^+$ T cells in N$^+$ nodes was associated with prolonged progression-free (PFS) and overall survival (OS). These data suggest that CD3$^+$CD8$^+$TIM-3$^+$ T cells may suppress tumor spread to regional lymph nodes but once tumor cells metastasize to lymph nodes, CD3$^+$/CD8$^+$/PD-1$^+$/Ki67$^+$ T cells localizing to N$^+$ nodes may prevent further tumor spread, resulting in prolonged survival.

Keywords: NSCLC, metastatic lymph node, T cell subsets, PD-1, TIM-3

Introduction

Lung cancer accounts for approximately 18.4% of all cancer deaths worldwide [1], in part due to its frequent presentation at advanced stage where long-term survival rates are less than 5% [2]. Intratumoral heterogeneity has been one of the major obstacles to developing curative treatments [3], but also offers opportunities for the use of immunotherapy [4-6] because the high tumor mutational load of many lung cancers results in a greater number of neoantigens available as targets for cognate cytolytic T cells [7]; however, T cells stimulated against tumor antigens rapidly express PD-1 [8] which upon interaction with PD-L1 and PD-L2 on tumor cells or other infiltrating immune cells, triggers T cell dysfunction [9]. These CD8$^+$PD-1$^+$ tumor infiltrating lymphocytes (TILs), though, have the capacity to recover reactivity, resulting in higher tumor-specific IFN-γ production compared with CD8$^+$PD-1$^-$ T cells [8]. TIM-3 expression is also frequent among CD8$^+$ T cells in the tumor immune microenvironment and correlates with infiltrating IFNγ-producing T cells [10-14]. A recent analysis of TILs in resected NSCLC observed that while PD-1 and TIM-3 expression were associated with each other, they had e microenvironment and correlates withnctional implications, and genomic correlates" [15].

Therefore, $CD3^+CD8^+PD\text{-}1^+$ and $CD3^+CD8^+TIM\text{-}3^+$ T cells may have only partially overlapping functions in the host response to malignant cells in the tumor stroma.

Lymph nodes are sites where dendritic cells (DC) bearing tumor antigens activate T cells with receptors that recognize these antigens. Immune cell trafficking to lymph nodes depends on chemokines and adhesion molecules; however, many of these molecules participate in the process of tumor metastasis [16]. Lung cancers often metastasize initially to hilar or interlobar lymph nodes (N_1) close to the tumor, but they may also spread to ipsilateral (N_2) or contralateral (N_3) mediastinal lymph nodes [17] events which are independent predictors of poor prognosis [17]. The immune response within the primary tumor tissue has a well-established association with outcome [18], but less is known about the roles or effects of immune effectors in regional lymph nodes draining primary tumors. Further, differences in the function of T cells distributed within lymph nodes containing tumor metastases (N^+) compared with tumor uninvolved lymph nodes (N^-) have not been well described. In the present study, we have explored the frequency of various T cell subtypes in the adjacent lymph nodes of surgically resected NCSLC, both N^+ and N^-, from the specimens of patients with N1 disease and the N^- nodes of those with N0 disease. We wished to address whether certain phenotypes, and in particular, PD-1 and TIM-3 expression by T cells, were associated with clinical outcome. The specific T cell populations exposed to different phases of tumor invasion into lymph node were identified as well.

Methods

Patients and sample preparation

The study was approved by the Institutional Review Committee of Beijing Shijitan Hospital, Capital Medical University Cancer Center. Primary tumors and regional lymph nodes obtained from NSCLC surgically resected between August 2012 and August 2019 and fixed in formalin and paraffin embedded (FFPE) were selected for analysis. This resulted in a total of 54 lymph nodes (27 N^+ and 15 N^- collected from 12 patients with Stage IIB ($T_2N_1M_0$), and 12 N^- from 10 cases with Stage IIA ($T_2N_0M_0$)) available. The surgical records and pre-operative radiological images were used to re-stage the tumors according to the eighth edition of the International Union Against Cancer TNM classification [19]. Diagnoses of tumor metastasis lymph nodes were confirmed from hematoxylin and eosin-stained slides by a pathologist (P.S.). The histological classification was done according to the 2015 World Health Organization Classification of Lung Tumors [20]. Seven of the twelve stage IIB cases included both primary tumor samples and interlobar lymph node samples from the lobectomy plus lymph node dissection. There were 3 cases whose interlobar lymph node tissue was obtained from endobronchial ultrasound guided-transbronchial needle aspiration (EBUS-TBNA). Clinical outcomes (PFS, OS) were determined by chart review. Histology, clinical stage, and analyzed lesions are summarized in **Table 1**.

Multiple color immunofluorescence staining and antibody selection

In order to perform multi-label staining analysis, we applied histiocytometry (multiple quantitative tissue imaging analysis) to all samples as we previously described. The slides with lung cancer samples were deparaffinized in xylene and rehydrated in a graded series of ethanol solutions. Antigen retrieval was performed by microwaving the slides in citrate buffer (pH6) at 95°C for 20 minutes, and then cooling at room temperature for 20 minutes. After quenching the endogenous peroxidase in 3% H_2O_2, the incubated slides were covered with the blocking agent (ZSGB-BIO, ZLI-9022) at room temperature for 30 minutes. We followed the Opal technology instructions to complete the multiple color staining using a 7-color IHC kit (NEL797001KT, 1:100). Briefly, the slides were incubated with each primary antibody for 2 hours in a humid chamber at 37°C, and then a secondary antibody conjugated with HRP (GBI Labs, Polink-1 HRP polymer detection kit) followed by TSA-fluors (PerkinElmer, Opal) and microwaving in a citrate buffer (pH 6.0) at 95 g agent (ZSGB-BIO, ZLI-9022) at room temperature for 30 minutbeled with a different fluorophore. The cell nucleus was then illuminated with DAPI (1:2000), and ProLong Gold Antifade Mountant (ThermoFisher, P36934) and cover glass were applied. Multiplex antibody panels used in this study are: panel 1: PD-1 (CST, 43248, 1:200, *Opal* 520), CD3 (Abcam

Table 1. Patient demographics, staging, and therapy

Patient	Sex	Age	Pathology	Differentiation	Primary tumor lesion[#]	Total number of lymph node[*]	Number of metastatic lymph nodes	Postoperative chemo-therapy	Chemotherapy cycle	TNM staging	Tumor staging	Drinking history[&]	Smoking history[&]	PFS (Days)	OS (Month)
NO. 1	Male	55	Adenocarcinoma	Well-differentiated	Y	5	3	Pemetrexed + Cisplatin	4	T2N1M0	IIB	Y	Y	1122	47
NO. 2	Male	66	Acinar adenocarcinoma	Well and moderately differentiated	N	3	1	Pemetrexed + Cisplatin	3	T2N1M0	IIB	Y	N	305	+
NO. 3	Male	57	Adenocarcinoma	Well and moderately differentiated	Y	5	4	Pemetrexed + Cisplatin	3	T2N1M0	IIB	N	N	330	14
NO. 4	Male	79	Adenocarcinoma	Well-differentiated	Y	8	7	Pemetrexed + Cisplatin	4	T2N1M0	IIB	Y	Y	611	28
NO. 5	Male	77	Adenocarcinoma	Well-differentiated	N	2	1	Paclitaxel + Cisplatin	4	T2N1M0	IIB	N	Y	182	8
NO. 6	Female	65	Adenocarcinoma	Well and moderately differentiated	Y	2	1	Pemetrexed + Cisplatin	4	T2N1M0	IIB	N	N	305	22
NO. 7	Male	57	Adenocarcinoma	Well-differentiated	Y	3	1	Paclitaxel + Cisplatin	4	T2N1M0	IIB	N	N	1797	69
NO. 8	Male	45	Adenocarcinoma	Well-differentiated	Y	3	2	Pemetrexed + Cisplatin	4	T2N1M0	IIB	Y	Y	225	12
NO. 9	Female	59	Adenocarcinoma	Well and moderately differentiated	Y	3	2	Pemetrexed + Cisplatin	4	T2N1M0	IIB	N	N	1432	55
NO. 10	Male	58	Adenocarcinoma	Well-differentiated	N	2	1	Pemetrexed + Cisplatin	3	T2N1M0	IIB	Y	N	883	+
NO. 11	Male	62	Adenocarcinoma	Well and moderately differentiated	Y	3	2	Paclitaxel + Cisplatin	3	T2N1M0	IIB	N	N	321	32
NO. 12	Male	61	Adenocarcinoma	Well-differentiated	Y	3	2	Pemetrexed + Cisplatin	4	T2N1M0	IIB	Y	Y	149	15
NO. 13	Female	52	Adenocarcinoma	Well and moderately differentiated	Y	1	-	Pemetrexed + Cisplatin	4	T2N0M0	IIA	N	N	$	-
NO. 14	Male	68	Adenocarcinoma	Well-differentiated	Y	1	-	Pemetrexed + Cisplatin	3	T2N0M0	IIA	Y	Y	557	35
NO. 15	Male	49	Adenocarcinoma	Well and moderately differentiated	Y	1	-	Pemetrexed + Cisplatin	4	T2N0M0	IIA	Y	Y	792	46
NO. 16	Female	52	Adenocarcinoma	Well-differentiated	Y	2	-	Pemetrexed + Cisplatin	4	T2N0M0	IIA	N	N	$	-
NO. 17	Male	57	Adenocarcinoma	Well-differentiated	Y	1	-	Pemetrexed + Cisplatin	4	T2N0M0	IIA	N	N	$	-
NO. 18	Male	60	Adenocarcinoma	Well and moderately differentiated	Y	1	-	Pemetrexed + Cisplatin	4	T2N0M0	IIA	Y	N	991	61
NO. 19	Female	66	Adenocarcinoma	Well-differentiated	Y	1	-	Pemetrexed + Cisplatin	4	T2N0M0	IIA	N	N	$	-
NO. 20	Female	59	Adenocarcinoma	Well-differentiated	Y	2	-	Pemetrexed + Cisplatin	4	T2N0M0	IIA	Y	N	1956	78
NO. 21	Male	48	Adenocarcinoma	Well-differentiated	Y	1	-	Pemetrexed + Cisplatin	4	T2N0M0	IIA	N	N	2133	71
NO. 22	Female	62	Adenocarcinoma	Well and moderately diferentiated	Y	1	-	Pemetrexed + Cisplatin	4	T2N0M0	IIA	N	Y	578	21

*: Number of metastatic lymph nodes and corresponding drainage area of normal lymph node found in tissues after lymph node dissection. #: N represent the patient only had N^{+} through EBUS-TBNA . Y represent the patient had primary lesion through lobectomy plus lymph node dissection. &: N represent no history of smoking or drinking, Y represent had a history of smoking or drinking. +: These patient was lost after the progress. $: No progress occurred until the end of the follow-up date.

ab16669, 1:100, *Opal* 430); EGFR (Abcam ab52894, 1:200, *Opal* 560), TIM3 (CST, 45208, 1:200, *Opal* 610), Ki67 (Abcam, ab92742, 1:400, *Opal* 650), CD8 (DAKO, M710301-2, 1:200, *Opal* 700); panel 2: CXCR4 (Abcam ab12-4824, 1:200, Opal 520), PD-L1 (CST, 13684, 1:100, Opal 610), Foxp3 (Abcam, ab20034, 1:100, *Opal* 650), CD4 (Abcam ab133616, 1:500, *Opal* 700), PD-1 (CST, 43248, 1:200, *Opal* 560).

Digital image acquisition and analysis

We applied the TissueFAXS slide scanning system (Tissue Gnostics) based on the Zeiss Axio Imager Z2 vertical epi-fluorescence microscope to scan the fluorescently labeled slides. At the same time, we used Zeiss 20 Plan Apochromat air objective (0.8 NA) to capture the image. TissueQuest software (TissueGnostics) was used to analyze the fluorescence values. The DAPI-stained nuclei were segmented, and thresholds set for different intensities were used to identify all DAPI nuclei. We used the TissueQuest analysis platform to determine the number of cells and the average intensity, in order to determine the percentage of positive cells relative to the total number of cells (percentage of positive cells/all nucleated cells) and the cell density as previously described [21].

Statistical analysis

The fluorescence intensity of each pixel reflects the expression level of the marker corresponding to the fluorescence value. We calculated the number of each marker expressed by each cell in the representative field. The ratio of the number of cells positive for the specific marker to the total number of cells is also calculated. All data are expressed as mean +/- SEM. We used GraphPad Prism software for statistical analysis. The unpaired two-tailed Student's t-test was used to calculate *P* values (*: P<0.05; **: P<0.001; ***: P<0.0001; ns, not significant).

Results

Patient characteristics

Patient demographics, clinical parameters, and tumor stage for each of the 22 cases are shown in **Table 1**. The mean age of patients was 59 years (range 45-79 years). 36 % of patients reported a history of smoking. All patients had undergone surgical resection of NSCLC followed by chemotherapy which consisted of cisplatin plus pemetrexed in 80%. Five patients received only 3 cycles of chemotherapy because of adverse reaction of severe myelosuppression. According to the TNM classification criteria, all tumors were T2. Twelve cases were N1 and ten were N0. The individual PFS and OS times are listed in **Table 1**.

T cell distributions in primary tumors and lymph nodes of N1 compared with N0 patients

We compared the frequency of various cytolytic T cell subsets ($CD3^+/CD8^+/PD\text{-}1^+/TIM\text{-}3^+$ T cells) in primary tumors and draining lymph nodes of patients with and without lymph node metastases. The frequency of $CD3^+CD8^+TIM\text{-}3^+Ki\text{-}67^+$ T-cells (P<0.0001) was markedly higher in the Stage IIA primary tumors, and there was no statistical difference in the frequency of the other various lymphoid subtypes in the primary tumors of Stage IIB compared with Stage IIA patients (**Figure 1A**). In contrast, the frequency of $CD3^+CD8^+$ (P=0.0001), $CD3^+CD8^+$ $TIM\text{-}3^+$ (P<0.0001) and $CD3^+CD8^+TIM\text{-}3^+Ki\text{-}67^+$ (P<0.0001) T-cells was markedly higher in the uninvolved lymph nodes of N0 patients, than the lymph nodes of Stage IIB patients, all others being the same (**Figure 1B**). Further, the frequency of $CD3^+CD8^+$ (P=0.004), $CD3^+CD8^+$ $TIM\text{-}3^+$ (P<0.0001), $CD3^+CD8^+PD\text{-}1^+Ki\text{-}67^+$ (P=0.0075) and $CD3^+CD8^+TIM\text{-}3^+Ki\text{-}67^+$ (P<0.0001) T cells in N0 lymph nodes was also significantly higher than in the non-metastatic lymph nodes of Stage IIB patients (**Figure 1C**). These data suggest that in patients with lymph node metastases (N1+), there is a decrease in activated cytolytic T cell subsets in lymph nodes regardless of whether involved (N^+) or uninvolved (N^-).

T cell distributions in involved and uninvolved lymph nodes and the primary tumor of N1 patients

In order to study in greater detail the T cell composition in lymph nodes with and without metastases in Stage IIB patients, we analyzed the composition of different cytotoxic ($CD3^+/CD8^+/PD\text{-}1^+/TIM\text{-}3^+$ T cell) and suppressive ($CD4^+/Foxp3^{+/}PD\text{-}1^+$ T cell) lymphocyte subsets in the primary tumor tissues and lymph nodes

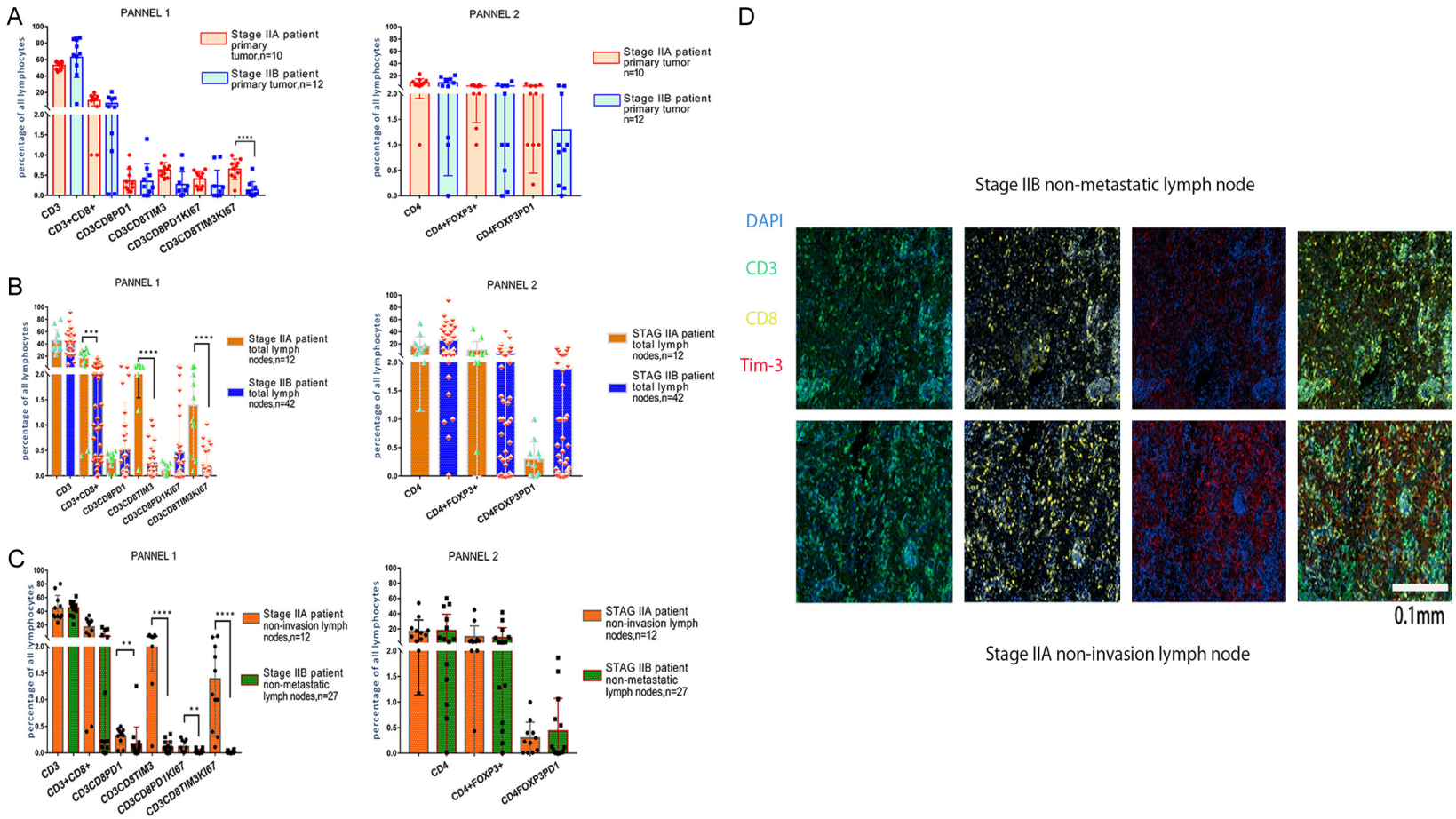


Figure 1. T cell distributions in primary tumor and lymph nodes of patients with Stage N0 and Stage N1 NSCLC. A: Lymphocyte subsets in Stage IIA patients' primary tumors compared with Stage IIB patients' primary tumors. B: Lymphocyte subsets in Stage IIA patients' lymph nodes compared with the tumor-involved lymph nodes of Stage IIB patients. C: Lymphocyte subsets in Stage IIA patients' lymph nodes compared with Stage IIB patients' non-metastatic lymph nodes. D: Example of immunofluorescent staining of lymph node sections for the indicated markers. (DAPI antibody is expressed in blue, CD3 antibody is expressed in green, CD8 antibody is expressed in yellow, and Tim-3 antibody is expressed in red).

of all 7 Stage IIB cases. In a comparison of T cell composition of the primary tumor tissues and involved lymph nodes (N+), only the expression of CD3$^+$CD8$^+$PD-1$^+$Ki-67$^+$ T-cells in the metastatic lymph nodes was markedly higher than that in primary tumor tissues (P=0.0081), all others being the same (**Figure 2A**). The lymphocyte subset composition was then compared between involved and uninvolved lymph nodes from each patient. CD3$^+$CD8$^+$PD-1$^+$ (P= 0.0086), CD3$^+$CD8$^+$TIM-3$^+$ (P=0.01) and CD3$^+$ CD4$^+$Foxps3$^+$PD-1$^+$ T-cells (P=0.026) were more frequent in the metastatic than non-metastatic lymph nodes (**Figure 2B**). High frequencies of proliferating CD3$^+$CD8$^+$PD-1$^+$Ki-67$^+$ T cell (P< 0.0001) and CD3$^+$CD8$^+$TIM3$^+$Ki67$^+$ T-cells (P= 0.0001), were also observed in the metastatic lymph nodes. These data suggest that both an effector T cell and suppressive T cell population are activated in response to tumor metastases in regional lymph nodes.

T cell and tumor cell phenotype in metastatic lymph nodes varies between involved lymph nodes compared with primary lesions

Lung cancer has considerable inter- and intra-patient molecular heterogeneity such that metastatic lymph nodes may have molecular features that differ from primary lesions [22-24]. We hypothesized that the immune reactions stimulated by such molecularly heterogeneous tumors would also be varied. To understand whether there was consistency among the lymphocyte subsets in each lymph node involved with tumor of stage IIB patients, the relatively common EGFR molecule that is frequently expressed in non-small cell lung cancer (NSCLC) and the CXCR4 and PD-L1 molecules that are associated with the immune microenvironment were used to label tumor cells, so as to observe the expression consistency of tumor molecules in 7 patients with primary tumor lesions and more than two metastatic lymph nodes, as well as the changes in lymphocyte subsets. The expression pattern of EGFR, CXCR4 and PD-L1 and the T lymphocyte subsets varied between patients and within patients (**Figure 3**). For example, the EGFR expression in two lymph nodes of patient 1 was higher but in one lymph node was lower than the primary tumor lesion. One involved lymph node had higher CD3$^+$CD8$^+$ T cells and fewer CD3$^+$CD8$^+$PD-1$^+$ and CD3$^+$CD8$^+$TIM3$^+$ T cells than other involved lymph nodes.

Among the 9 patients with resections of both the primary tumor and lymph nodes, 4 of 9 had a higher number of cytotoxic lymphocytes (CD3$^+$/CD8$^+$/PD-1$^+$/TIM-3$^+$ T cell) in metastatic lymph nodes compared with the primary lesions (**Figure 4A**). However, 5 of 9 had the reverse scenario--fewer cytotoxic T lymphocytes in the metastatic lymph node than primary tumor (**Figure 4B**). The PFS was markedly better for those with a greater frequency of cytotoxic T cells in the metastatic lymph nodes than the primary tumor (P=0.0049, median PFS: 1122 days *vs* 305 days, HR=0.07 95% CI: 0.01-0.4) (**Figure 4C**).

Frequency of peritumoral T cell subsets varies in N$^+$ and primary lesion and is associated with clinical outcome

The median percentage of cytotoxic (CD3$^+$/ CD8$^+$/PD-1$^+$/TIM-3$^+$ T cell) and suppressive (CD4$^+$/Foxp3$^+$/PD-1$^+$ T cell) lymphocytes across all metastatic lymph nodes was calculated for each patient. When each lymphocyte subset was analyzed across all 12 N$^+$ patients, the median frequencies were (CD3$^+$ T cell: 0.42%, CD3$^+$CD8$^+$ T cell: 0.033%, CD3$^+$CD8$^+$PD-1$^+$ T cell: 0.005%, CD3$^+$CD8$^+$PD-1$^+$Ki-67$^+$ T cell: 0.003%, CD3$^+$CD8$^+$TIM-3$^+$ T cell: 0.003%, CD3$^+$ CD8$^+$TIM-3$^+$Ki-67$^+$ T cell: 0.002%, CD4$^+$Foxp3$^+$ T cell: 0.18%, CD4$^+$Foxp3$^+$PD-1$^+$ T cell: 0.025%). Progression free and overall survival were greater amongst those with involved lymph nodes containing more CD3$^+$CD8$^+$ T cells (median PFS in positive: 866 days *vs* 265 days in negative group, P=0.03; median OS in positive: 40 months vs 15 months in negative group, P=0.035), more CD3$^+$CD8$^+$PD-1$^+$ T cells (median PFS in positive: 1122 days *vs* 305 days in negative group, P=0.0057; median OS in positive: 47 months *vs* 15 months in negative group, P=0.0028) and more CD3$^+$CD8$^+$PD-1$^+$KI-67$^+$ T cell (median PFS in positive: 1122 days *vs* 305 days in negative group, P=0.001; median OS in positive: 47 months vs 15 months in negative group, P=0.0033). Fewer CD4$^+$ Foxp3$^+$ T cells in the metastatic lymph nodes was associated with prolonged overall survival (median OS in positive: 14 months *vs* 32 months in negative group, P=0.037) and there was a non-significant effect on the progression-

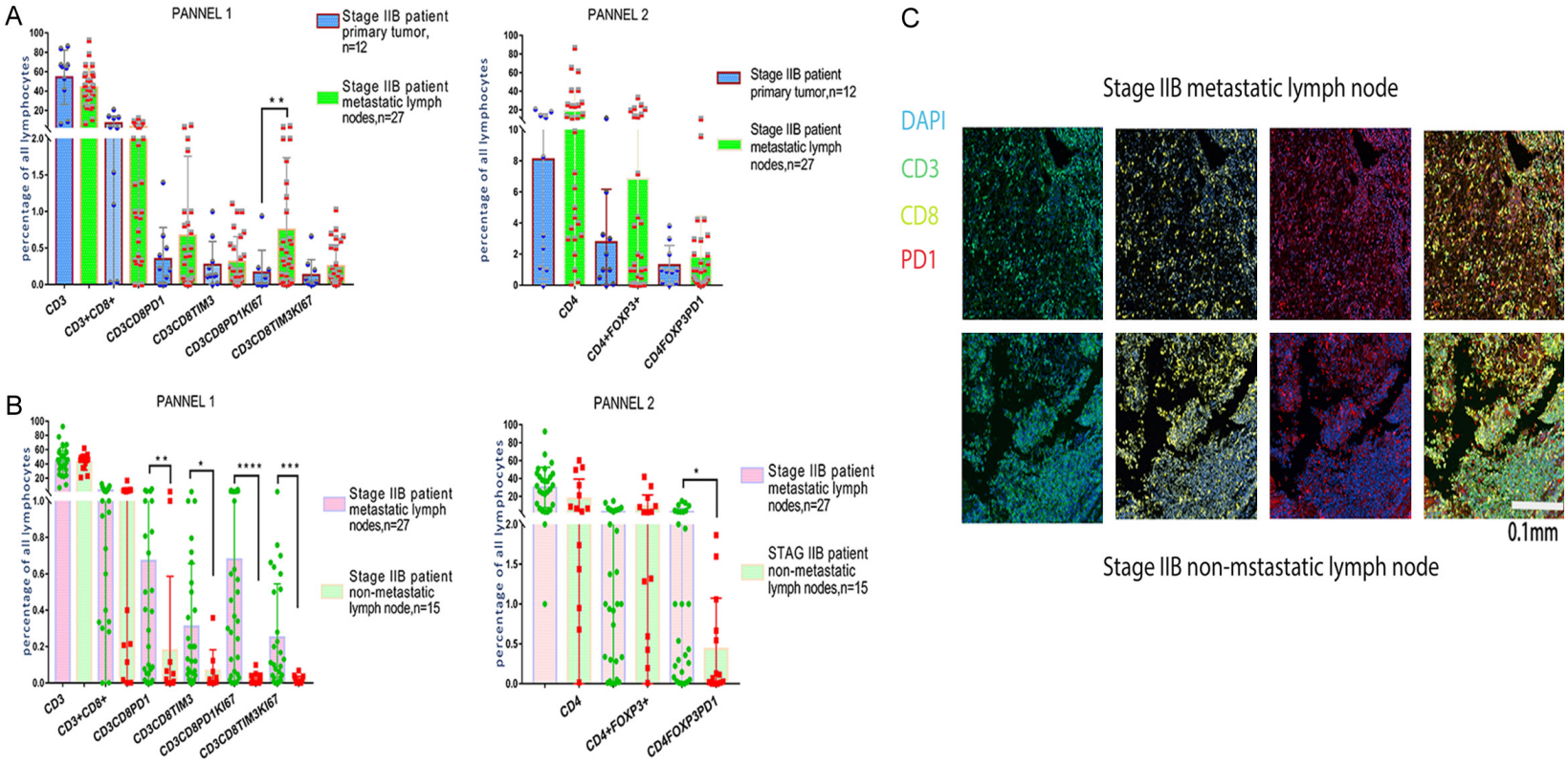
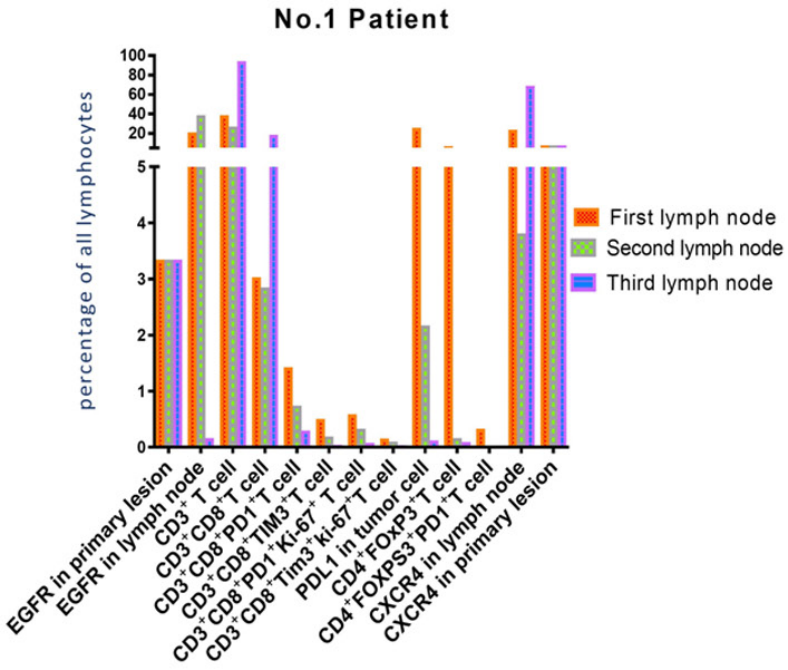
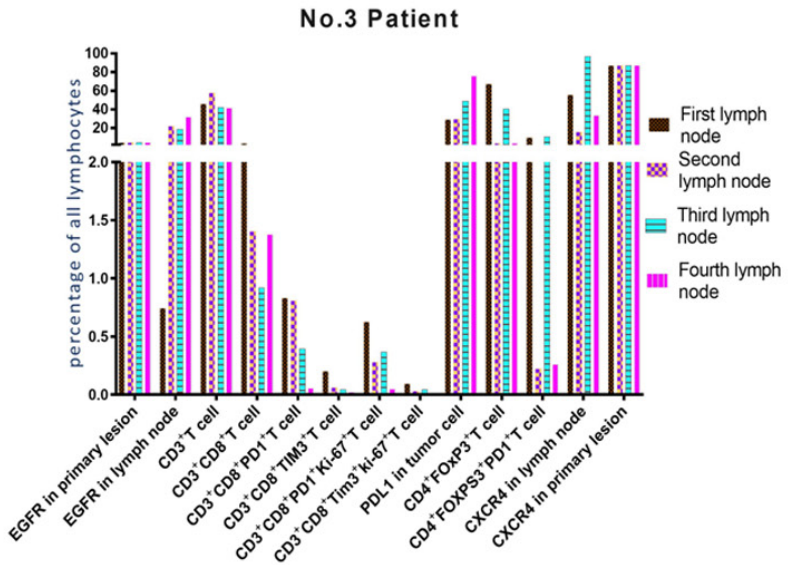
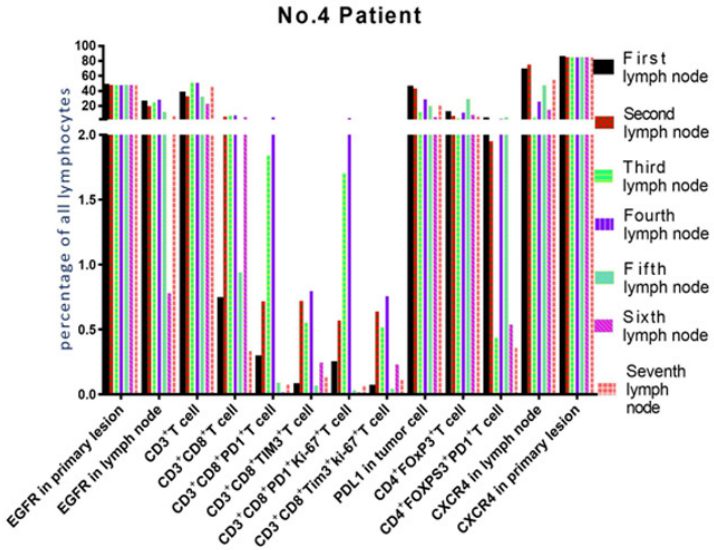
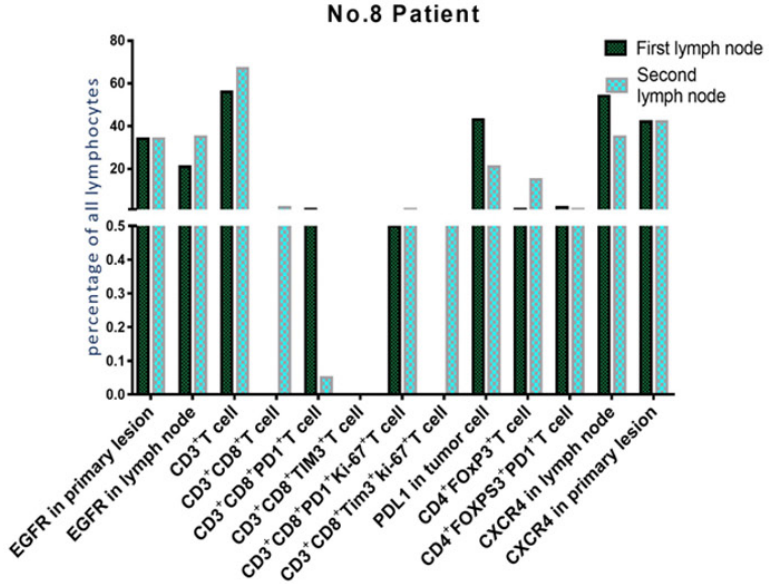


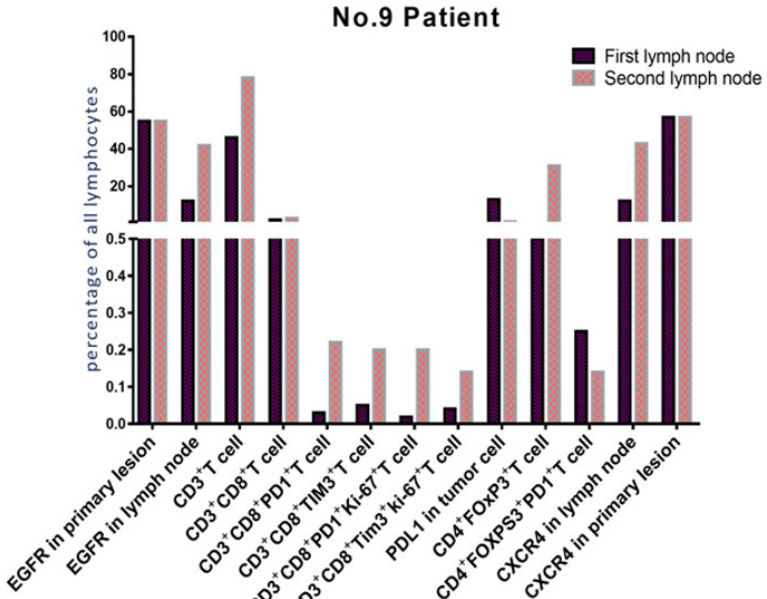
Figure 2. Lymphocyte subpopulations in primary tumor, involved and uninvolved lymph nodes. A: Lymphocyte subsets in involved lymph nodes (N^+) compared with the primary tumor. B: Lymphocyte subsets in involved lymph nodes (N^+) compared with uninvolved lymph nodes (N^-) C: Example of immunofluorescent staining of lymph node sections for the indicated markers. (DAPI antibody is expressed in blue, CD3 antibody is expressed in green, CD8 antibody is expressed in yellow, and PD-1 antibody is expressed in red).

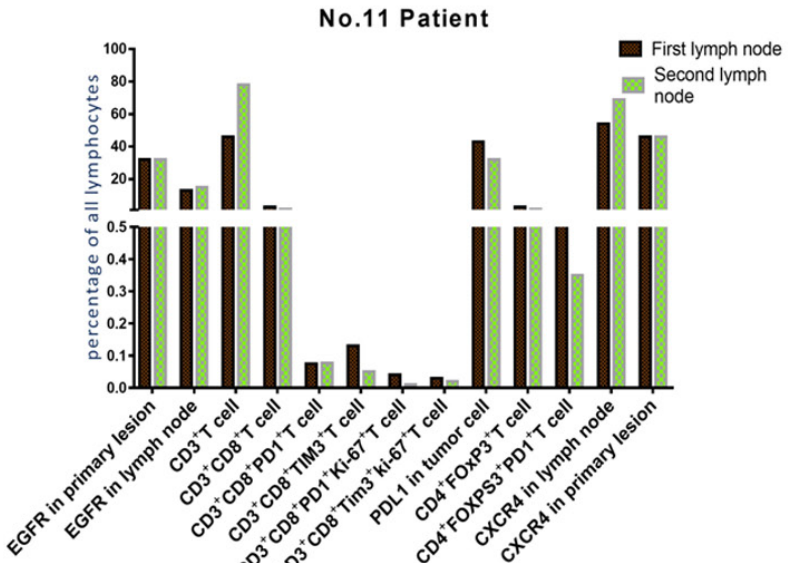

No.1 Patient
percentage of all lymphocytes
First lymph node
Second lymph node
Third lymph node
EGFR in primary lesion
EGFR in lymph node
CD3+ T cell
CD3+CD8+T cell
CD3+CD8+PD1+T cell
CD3+CD8+TIM3+T cell
CD3+CD8+PD1+Ki-67+T cell
CD3+CD8+Tim3+ki-67+T cell
PDL1 in tumor cell
CD4+FOxP3+T cell
CD4+FOXPS3+PD1+T cell
CXCR4 in lymph node
CXCR4 in primary lesion


No.3 Patient
percentage of all lymphocytes
First lymph node
Second lymph node
Third lymph node
Fourth lymph node
EGFR in primary lesion
EGFR in lymph node
CD3+T cell
CD3+CD8+T cell
CD3+CD8+PD1+T cell
CD3+CD8+TIM3+T cell
CD3+CD8+PD1+Ki-67+T cell
CD3+CD8+Tim3+ki-67+T cell
PDL1 in tumor cell
CD4+FOxP3+T cell
CD4+FOXPS3+PD1+T cell
CXCR4 in lymph node
CXCR4 in primary lesion


No.4 Patient
percentage of all lymphocytes
First lymph node
Second lymph node
Third lymph node
Fourth lymph node
Fifth lymph node
Sixth lymph node
Seventh lymph node
EGFR in primary lesion
EGFR in lymph node
CD3+T cell
CD3+CD8+T cell
CD3+CD8+PD1+T cell
CD3+CD8+TIM3+T cell
CD3+CD8+PD1+Ki-67+T cell
CD3+CD8+Tim3+ki-67+T cell
PDL1 in tumor cell
CD4+FOxP3+T cell
CD4+FOXPS3+PD1+T cell
CXCR4 in lymph node
CXCR4 in primary lesion


No.8 Patient
percentage of all lymphocytes
First lymph node
Second lymph node
EGFR in primary lesion
EGFR in lymph node
CD3+T cell
CD3+CD8+T cell
CD3+CD8+PD1+T cell
CD3+CD8+TIM3+T cell
CD3+CD8+PD1+Ki-67+T cell
CD3+CD8+Tim3+ki-67+T cell
PDL1 in tumor cell
CD4+FOxP3+T cell
CD4+FOXPS3+PD1+T cell
CXCR4 in lymph node
CXCR4 in primary lesion





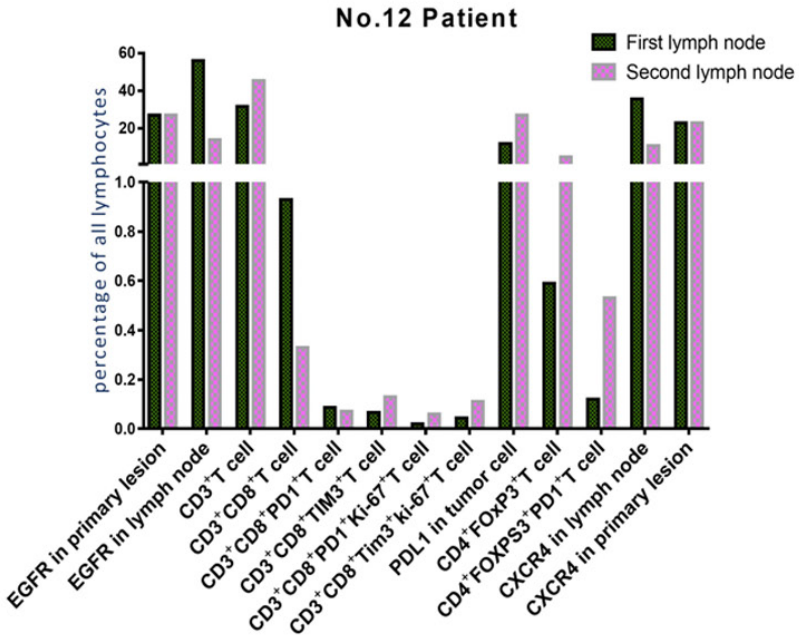


Figure 3. Lymphocyte subpopulations and molecular expression for involved lymph nodes and primary tumor for each patient. The lymphocyte subpopulations, and EGFR, PD-L1 and CXCR4 are presented for each tumor-involved lymph node and the primary tumor in each patient.

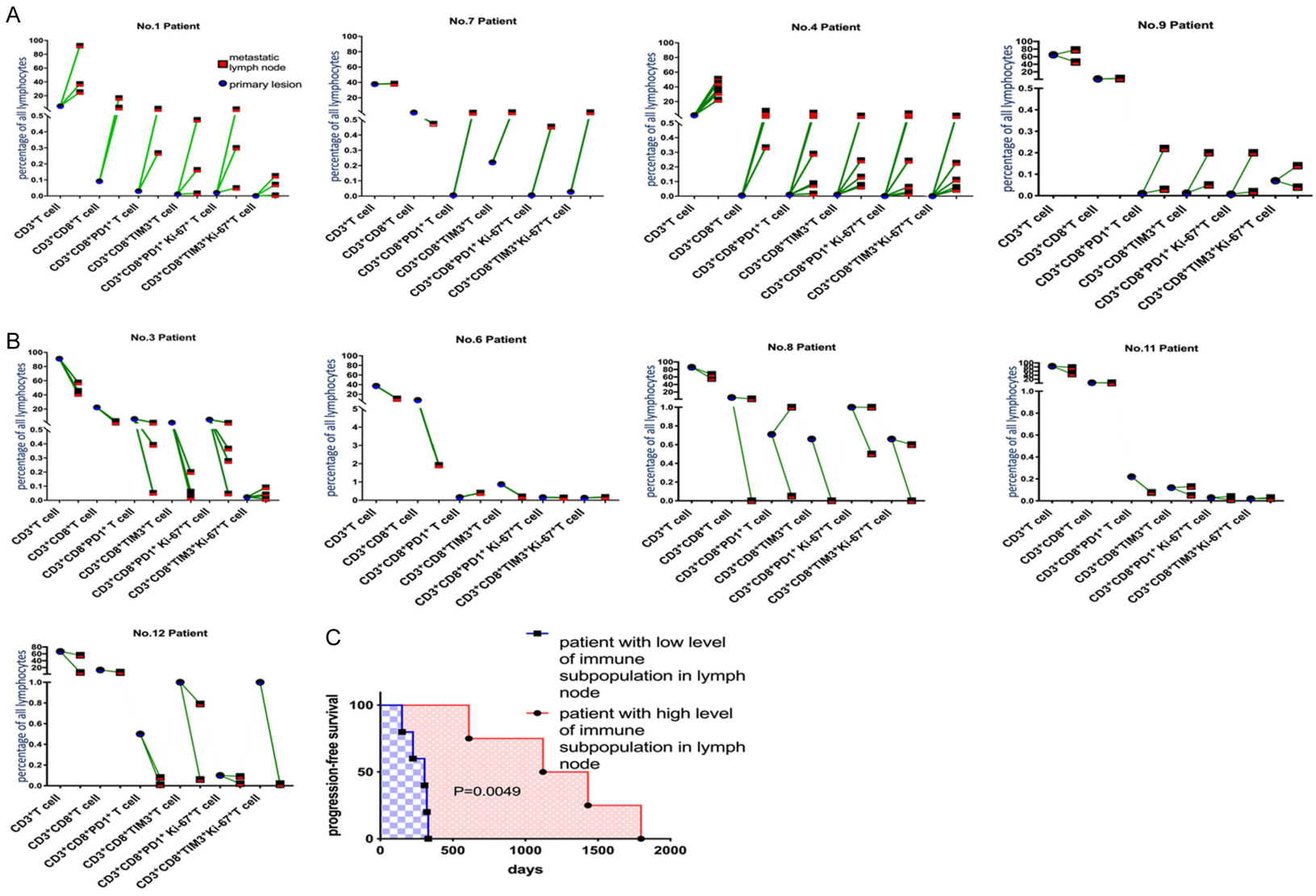
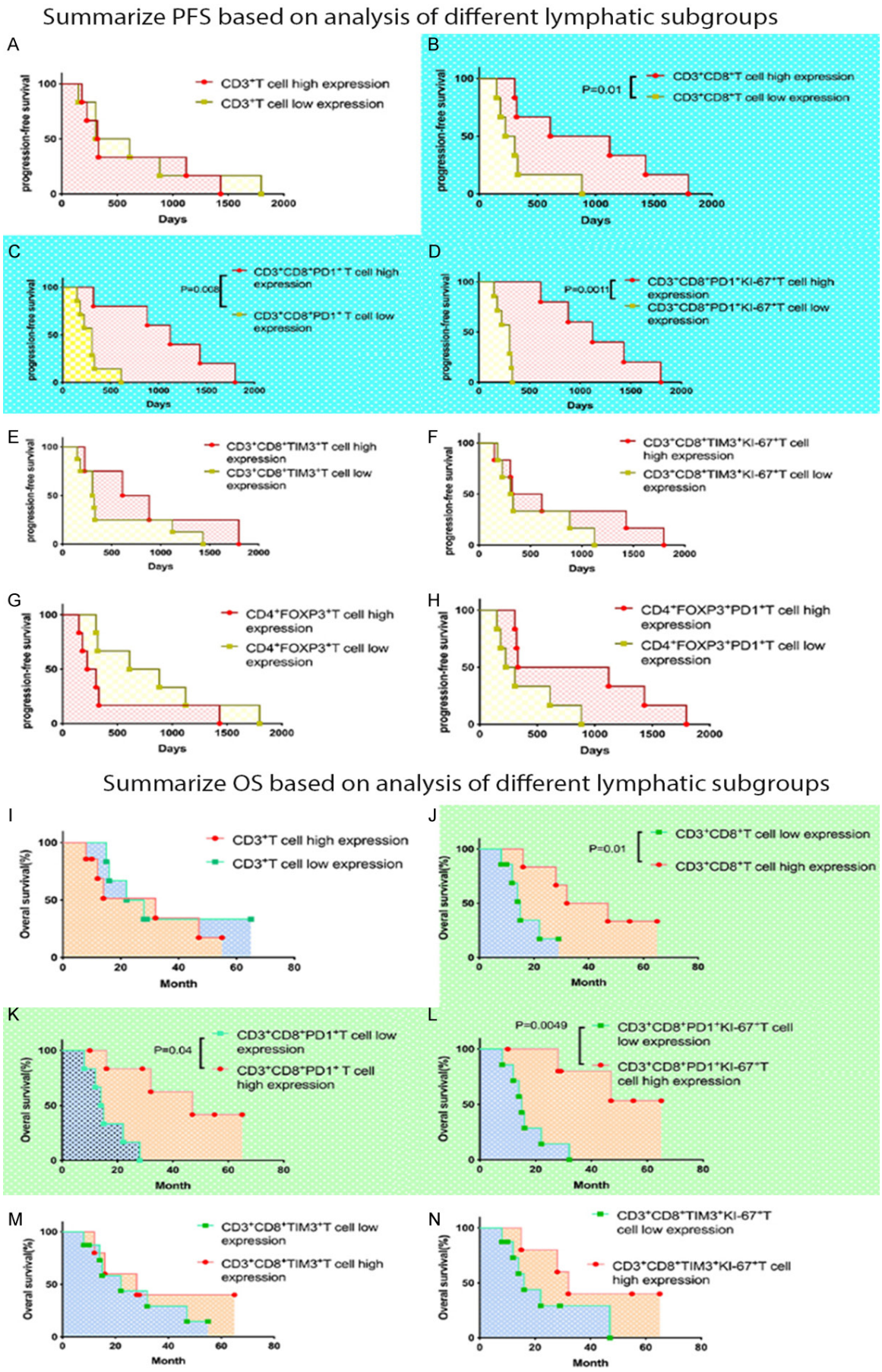


Figure 4. Lymphoid subsets in involved lymph nodes compared with primary tumor in each patient. A: Lymphocyte subsets in tumor and involved lymph nodes for the 4 patients for whom the frequency of these subsets was greater in lymph nodes. B: Lymphocyte subsets in tumor and involved lymph nodes for the 5 patients for whom the frequency of these subsets was lower in lymph nodes. C: The PFS for the two patient populations (greater or fewer lymphocyte subset frequencies in lymph nodes) (P=0.0049, median survival: 1122 days *vs* 305 days, HR=0.07, 95 CI: 0.01-0.4).


Summarize PFS based on analysis of different lymphatic subgroups
A
CD3+T cell high expression
CD3+T cell low expression
progression-free survival
Days
B
P=0.01
CD3+CD8+T cell high expression
CD3+CD8+T cell low expression
C
P=0.008
CD3+CD8+PD1+T cell high expression
CD3+CD8+PD1+T cell low expression
D
P=0.0011
CD3+CD8+PD1+KI-67+T cell high expression
CD3+CD8+PD1+KI-67+T cell low expression
E
CD3+CD8+TIM3+T cell high expression
CD3+CD8+TIM3+T cell low expression
F
CD3+CD8+TIM3+KI-67+T cell high expression
CD3+CD8+TIM3+KI-67+T cell low expression
G
CD4+FOXP3+T cell high expression
CD4+FOXP3+T cell low expression
H
CD4+FOXP3+PD1+T cell high expression
CD4+FOXP3+PD1+T cell low expression
Summarize OS based on analysis of different lymphatic subgroups
I
CD3+T cell high expression
CD3+T cell low expression
Overal survival(%)
Month
J
P=0.01
CD3+CD8+T cell low expression
CD3+CD8+T cell high expression
K
P=0.04
CD3+CD8+PD1+T cell low expression
CD3+CD8+PD1+T cell high expression
L
P=0.0049
CD3+CD8+PD1+KI-67+T cell low expression
CD3+CD8+PD1+KI-67+T cell high expression
M
CD3+CD8+TIM3+T cell low expression
CD3+CD8+TIM3+T cell high expression
N
CD3+CD8+TIM3+KI-67+T cell low expression
CD3+CD8+TIM3+KI-67+T cell high expression

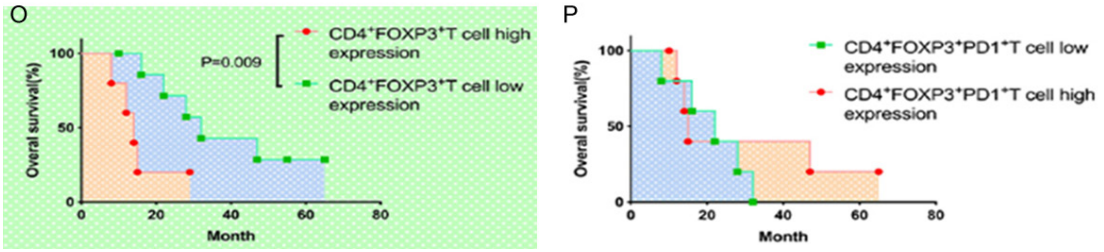


Figure 5. Survival analysis (PFS and OS) of subgroups divided by percentage of $CD3^+$ T cell, $CD3^+CD8^+$ T cell, $CD3^+CD8^+PD\text{-}1^+$ T cell, $CD3^+CD8^+PD\text{-}1^+Ki\text{-}67^+$ T cell, $CD3^+CD8^+TIM\text{-}3^+$ T cell, $CD3^+CD8^+TIM\text{-}3^+Ki\text{-}67^+$ T cell of the total cell. A, I: Patients with an increased percentage of $CD3^+$ T cells had non-significantly prolonged PFS and OS based on the cut-off value of 0.42%. B, J: Patients with an increased percentage of $CD3^+CD8^+$ T cells had significantly prolonged PFS and OS based on the cut-off value of 0.033%. C, K: Patients with an increased percentage of $CD3^+CD8^+PD\text{-}1^+$ T cells had a significantly prolonged PFS and OS based on the cut-off value of 0.005%. D, L: Patients with an increased percentage of $CD3^+CD8^+PD\text{-}1^+Ki\text{-}67^+$ T cells had significantly prolonged PFS and OS based on the cut-off value of 0.003%. E, M: Patients with an increased percentage of $CD3^+CD8^+TIM\text{-}3^+$ T cells had a non-significantly prolonged PFS and OS based on the cut-off value of 0.003%. F, N: Patients with an increased positive percentage of $CD3^+CD8^+TIM\text{-}3^+Ki\text{-}67^+$ T cells had a non-significantly prolonged PFS and OS based on the cut-off value of 0.002%. G, O: Patients with an increased percentage of $CD4^+Foxps3^+$ T cells had a non-significantly prolonged PFS and had a significantly prolonged OS based on the cut-off value of 0.18%. H, P: Patients with an increased percentage of $CD4^+Foxps3^+PD\text{-}1^+$ T cells had a non-significantly prolonged PFS and OS based on the cut-off value of 0.025%.

free survival. However other lymphoid subsets had no effect on PFS (as shown in the **Figure 5A**, **5E-5I**, **5M**, **5N**, **5P**, P>0.05). A higher frequency of proliferating $CD3^+CD8^+PD\text{-}1^+KI\text{-}67^+$ T cells was associated with better patient survival.

Discussion

The immune infiltrate of primary tumors has been extensively studied but less attention has been paid to the frequency and function of various lymphocyte subsets in draining lymph nodes. We applied the *Opal* Multiplex IHC method in N0 and N1 stage NSCLC to explore whether there were differences in various T cell subsets in tumor and adjacent lymph nodes comparing specimens from patients who had lymph node metastases and those who did not, and comparing the involved and uninvolved lymph nodes of patients with lymph node metastases. Because both PD-1 and TIM-3, while having some differences in their cellular distribution, both mark activated effector T-cells with cytolytic activity and reflect the status of antitumor immunity [25], we evaluated the $CD3^+CD8^+$ T cell subsets expressing these markers.

We found that $CD3^+/CD8^+/TIM\text{-}3^+/Ki\text{-}67^+$ T cells were enriched in the lymph nodes and in the primary tumor of patients with stage N0 compared with stage N1 NSCLC, but there were no significant differences in PD-1 expressing T cell subsets. These data suggest that in earlier stage lung cancers, TIM-3 but not PD-1 may mark an activated T cell subtype that prevents the development of lymph node metastases. A potentially paradoxical finding was that the frequency of $CD3^+CD8^+TIM\text{-}3^+Ki\text{-}67^+$ (P=0.00001) T cells was greater in the tumor involved (N^+) nodes of N1 patients compared with the tumor-uninvolved (N^-) nodes. Because TIM-3 delivers inhibitor signals to T cells, these data suggest that once tumor has spread to regional LNs, the inhibitory function of TIM-3 may predominate reducing the ability of TIM-3 + effector T cells to control the tumor.

In contrast to the scenario in stage N0 tumors, the relevant subtype in patients with stage N1 appears to be the PD-1 expressing T cell subset. This subtype has been found to have greater killing activity than that of PD-1-negative lymphocytes [26-28]. Further, based on the well-established intra-patient molecular heterogeneity of lung cancer [22-24], we hypothesized that the responding lymphocyte populations would also vary in frequency based on their varying migration to different lymph nodes (23) and this would be associated with clinical outcome. We had the opportunity to evaluate the primary tissue and associated metastatic (N^+) and non-metastatic (N^-) lymph nodes in stage N1 NSCLC patients, observing heterogeneity in expression of the EGFR molecule, CXCR4, and PD-L1 molecule across N^+ lymph

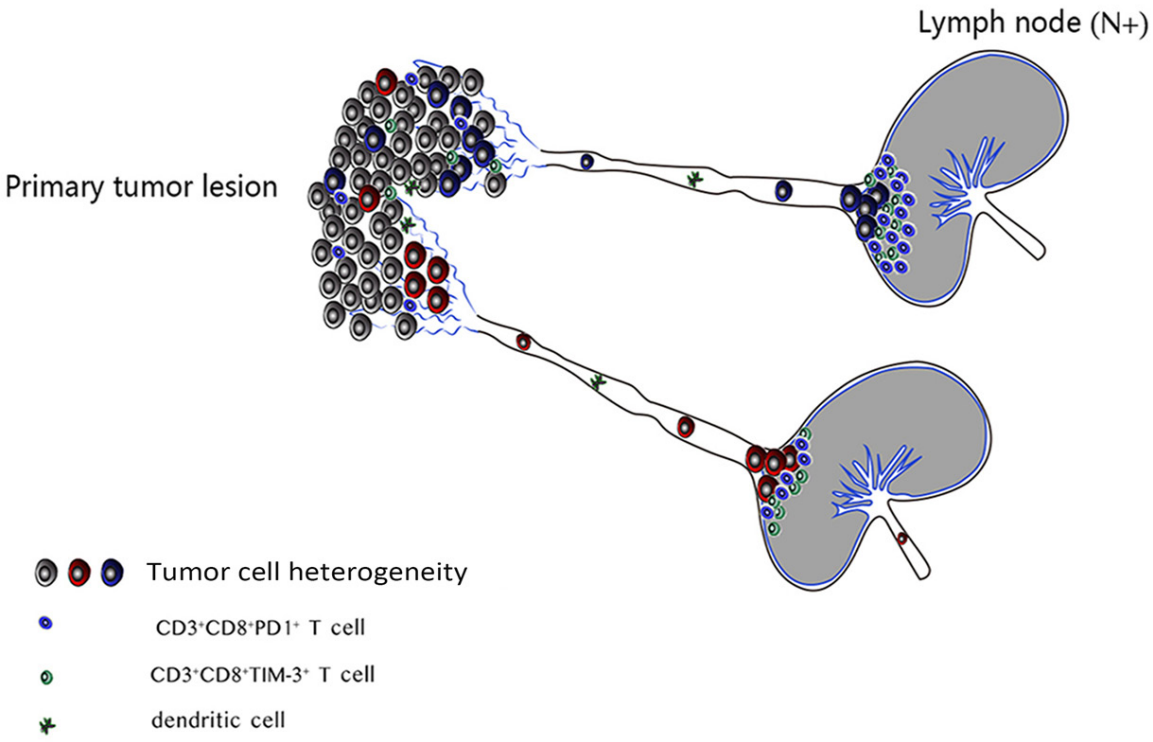


Figure 6. Progression of lymphatic metastasis from primary tumor to tumor-draining LN (N1). Primary tumors induce lymphangiogenesis to facilitate lymphatic metastasis. Cancer cells, T cells, and dendritic cells enter lymphatic capillaries and migrate through collecting lymphatic vessels to LNs. The subtypes of T cells that predominate at draining lymph nodes (e.g., $CD3^+CD8^+PD\text{-}1^+$ and $CD3^+CD8^+TIM\text{-}3^+$ T cells) may vary due to the heterogeneous expression of tumor molecules. Some metastatic lesions can cause a greater number of immunocompetent cells to aggregate, while others concentrate less immunologically active cells and are unable to eliminate cancer cells resulting in the development of distant metastases.

nodes. Similarly, the composition of the immune infiltrate varied across N^+ lymph nodes and also comparing N^+ and N^- lymph nodes and the primary tumor and N^+ lymph nodes. The increased frequency of $CD3^+CD8^+PD\text{-}1^+Ki\text{-}67^+$ T cell in the tumor-invaded lymph nodes suggests that the induction of antitumor immunity was strongest in the metastatic lymph nodes of N1 patients. In order to assess the clinical effect of the various T cell subsets on outcome, different cytotoxic and suppressive lymphocyte subsets were grouped into high expression and low expression groups, respectively, and we found that, $CD3^+CD8^+$ T cell, $CD3^+CD8^+PD\text{-}1^+$ T cell and $CD3^+CD8^+PD\text{-}1^+Ki\text{-}67^+$ T-cells with strong killing activity were associated with longer patient survival if they were highly expressed in metastatic lymph nodes. Taken together, these data suggest that $PD\text{-}1^+$ effector T cells infiltrate tumor involved lymph nodes and may play a major role in preventing further tumor metastasis.

Our proposed model is depicted in **Figure 6**. In stage N1 NSCLC, regional lymph nodes metastases occur, there is a shift to infiltration with $CD3^+CD8^+PD\text{-}1^+$ T cells, the presence of which are associated with improved prognosis. These data suggest that immune cells are present in NSCLC patients which recognize tumor and could be activated for use as an adjuvant therapy or in advanced disease. Indeed, we have reported on the activity of cytokine induced killer cells in advanced NSCLC patients [29]. Our data also suggest that attempts to augment the immune response prior to surgical resection of NSCLC could lead to longer disease-free and overall survival.

Conclusions

$CD3^+CD8^+TIM\text{-}3^+$ T cells may suppress tumor spread to regional lymph nodes but once tumor has spread to lymph nodes, $CD3^+/CD8^+/PD\text{-}1^+/Ki67^+$ T cells localized within N^+ nodes may pre-

vent some tumor cells from invading further resulting in prolonged survival.

Clinical practice points

Different T cell populations, marked by TIM-3 and PD-1, may be responsible for controlling tumor progression at different stages of NSCLC.

$CD3^+CD8^+TIM\text{-}3^+$ T cells may control tumor spread to regional lymph nodes but once tumor has spread to lymph nodes, $CD3^+/CD8^+/PD\text{-}1^+/Ki67^+$ T cells localized within N^+ nodes may prevent some tumor cells from invading further resulting in prolonged survival.

Immunotherapies that enhance the frequency or function of different T cell effector populations may have greater efficacy depending on tumor stage. For example, the use of non-specific DC-CIK may have greater efficacy in N0 disease but anti-PD(L)1 may be preferred when tumor has spread to lymph nodes.

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Disclosure of conflict of interest

None.

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