Foxp3$^+$ Regulatory T Cells and Natural Killer Cells Distinctly Infiltrate Primary Tumors and Draining Lymph Nodes in Pulmonary Adenocarcinoma

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Introduction: Regulatory T cells (Tregs) can play a key role in suppressing T-cell-mediated immunity in patients with cancer. In this study, the immune cell composition of the lung tissue and draining lymph nodes from patients with non-small cell lung cancer was analyzed.

Methods: Samples (solid tumor, tumor border, and tumor-free lung tissue, as well as intrapulmonal N1 and mediastinal N2 lymph nodes) from 30 patients subjected to curative resection were analyzed by immunohistochemistry and flow cytometry.

Results: Immunohistochemistry showed the presence of Foxp3$^+$ Tregs in tumor-infiltrated lung tissue, scattered Tregs in tumor-free lung samples, and a large number of these cells in metastatic lymph nodes. Using flow cytometry, we observed a significant enhancement of CD4$^+$ T cells and Foxp3$^+$ Tregs in the tumor center of adenocarcinoma samples, when compared with tumor-free lung tissues and tumor periphery. This enrichment was associated with a drastic decrease in natural killer cell amounts. Metastatic lymph nodes also showed higher Treg numbers than tumor-free ones in patients with lung adenocarcinomas. In contrast, patients with squamous cell carcinomas displayed less profound accumulation of Tregs.

Conclusion: Accumulation of Tregs in the center of lung tumors and in metastatic lymph nodes in combination with a decrease in the natural killer cell numbers suggests a critical role of Treg in the formation of immunosuppressive tumor microenvironment. Therefore, lung cancer immunotherapy may be improved by a specific Treg elimination or suppression.

Key Words: Foxp3$^+$ Tregs, NK-cells, Lung cancer, Lymph nodes, Tumor microenvironment.

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Lung cancer is the leading cause of cancer death worldwide, with a growing incidence in both developed and developing countries. Because the prognosis remains poor when using conventional cancer therapy methods, the need for new, effective adjuvant therapies is apparent.[1] Immunotherapy, in particular an activation of tumor-infiltrating immune cells, may offer a promising approach. Nevertheless, the success of such treatment has been limited up to now, probably due to the suppressive nature of the tumor. Indeed, most patients with cancer are not able to develop a satisfactory antitumor immune response, which implies the existence of effective tumor-specific immune evasion strategies, including the induction of tumor antigen-specific T-cell tolerance.[2] Regulatory T cells (Tregs) have attracted much interest, due to their crucial role in the suppression of self-antigen response.[2,3] On the other hand, lung tissue is known to harbor considerable numbers of natural killer (NK) cells, which are an important subset of antitumor effector cells.[4] Foxp3 has been reported as a key regulatory gene for the development and function of Tregs and is a commonly used marker of these cells.[5] In several solid cancers, elevated Treg proportions have been found in tumor tissue and peripheral blood; increased Treg numbers were also reported in tumor-infiltrated lymph nodes (TIL) in melanoma and gastrointestinal tumors.[6–9]

The aim of this study was to investigate proportions of Tregs and NK cells in primary lung tumors and in corresponding metastatic lymph nodes of the same patient in comparison with normal adjacent lung tissue. We showed a significant enhancement of Treg numbers in tumor tissue and metastatic lymph nodes. This Treg enrichment was associated with a drastic decrease in NK cells in tumor tissue. Our results suggest an important role of Treg in creating an immunosuppressive tumor microenvironment.

MATERIALS AND METHODS

Patients

A total of 30 patients (men: $n = 21$, women: $n = 9$; mean age: 63 years) receiving curative resection of non-small cell lung cancer (NSCLC) (adenocarcinoma: $n = 16$; squamous cell carcinoma: $n = 14$) were enrolled in this study; none was subjected to preoperative chemotherapy and/or

TABLE 1. Summary of the Clinical Data

	pT				pN			Stage			
Histopathology	**T1**	**T2**	**T3**	**T4**	**N0**	**N1**	**N2**	**1**	**2**	**3A**	**3B**
Squamous cell carcinoma	1	8	2	3	5	6	3	3	4	5	2
Adenocarcinoma	1	13	1	1	5	2	9	5	2	8	1
Total	2	21	3	4	10	8	12	8	6	13	3

radiotherapy. All patients underwent anterolateral thoracotomy, entering in the fourth or fifth intercostal space; lobectomy was performed in 17 patients, bilobectomy in three patients, and pneumonectomy in a total of 10 patients. Radical mediastinal and hilar lymphadenectomy was realized concurrently with all procedures, including four compartments in the right-sided thoracotomy (paratracheal, infracarinal, inferior mediastinal, and hilar) and four compartments in the left-sided thoracotomy (aortic, infracarinal, inferior mediastinal, and hilar).[10,11] The postoperative pathologic stage for lung cancer was determined according to the 7th edition of the classification of malignant tumors (TNM), revised in 2009.[12] The Ethics Committee of the University of Heidelberg approved this study (Study No. 270/2001). Each patient was informed comprehensively; written consent was given before participation. A synopsis of the clinical data is presented in Table 1.

Processing of Tissue Specimens

Immediately after surgical lung resection, tissue samples were extracted from three different locations. Tumor tissue was taken from a central area of solid tumor tissue lacking massive necrosis (tumor center). Tumor border included tissue closely adjacent and neighboring the tumor. For representative tumor-free lung tissue samples, tissue was taken from the surgically removed lung in the farthermost position to the tumor (healthy lung).

In 20 patients, lymph node tissue was taken from three locations: (i) an intrapulmonal lymph node (N1 location) in a tumor-draining position closest to the tumor, (ii) a mediastinal lymph node (N2 location) in a typical tumor-draining position (e.g., right upper mediastinal position in a right upper lobe tumor), and (iii) a mediastinal lymph node (N2 location) in a nontumor draining position (e.g., right lower mediastinal position in a right upper lobe tumor).

Immunohistochemistry

For routine histopathologic analysis, lung and lymph node tissues were embedded in paraffin and further processed for routine hematoxylin and eosin staining. Immunohistochemistry was performed to detect the localization of the $FoxP3^+$ lymphocytes in tumor, lung, and lymph node samples. Additional sections (1 μm thick) were cut, deparaffinized, and stained with mouse monoclonal antibodies (mAbs) against human Foxp3 (eBioscience) and CD4 (Dianova) using an autostaining device (Autostainer; DAKO, Glostrup, Denmark). Assays were performed according to the manufacturer's instructions. Immunohistochemical stainings were analyzed by two experienced pathologists (P.A.S. and A.W.).

Flow Cytometry

Biopsies of lung tissue and lymph node tissue were immediately transferred into serum-free Roswell Park Memorial Institute (RPMI) culture medium and stored on ice. After removal of necrotic tissue and fat, biopsies were cut into small pieces and filtered through a 100-μm nylon mesh. Samples were depleted of red blood cells by ammonium chloride lysis, washed twice with phosphate-buffered saline, and used for flow cytometry (FACS).

Single-cell suspensions were stained with directly conjugated mouse mAbs against human CD45-PerCP-Cy5.5, CD3-PerCP-Cy5.5, CD4-FITC, $CD8^-$ PE-Cy7, CD25 APC-

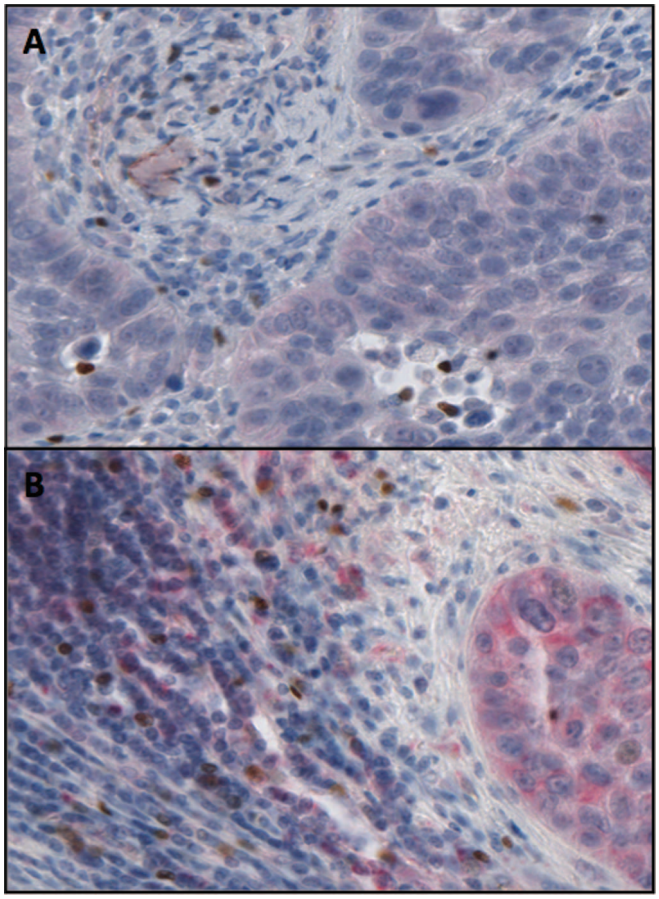

FIGURE 1. Regulatory T cell (Treg)-infiltrate primary tumors (*A*) and metastatic lymph nodes (*B*) in patients with lung cancer. Consecutive paraffin sections of 1 μm thickness were stained with Foxp3 (brown) monoclonal antibodies (mAbs) and counterstained with hemalaun.

H7, and CD56-PE or isotype-matched control mAbs (all from BD Bioscience, Franklin Lakes, NJ), fixed, and permeabilized (eBioscience) according to the manufacturer's protocol, followed by staining with Foxp3-APC (eBioscience, San Diego, CA). Acquisition was performed by four- or five-color flow cytometry using a FACS Canto II with FACSDiva software (BD Biosciences) with dead cell exclusion based on scatter profile. FlowJo software (Tree Star, Ashland, OR) was used to analyze at least 100,000 events. Data were expressed as dot plots.

RESULTS

Foxp3$^+$ Lymphocytes Infiltrate Primary Lung Tumors and Metastatic Lymph Nodes

Using immunohistochemistry, both peritumoral infiltration and intratumoral infiltration of FoxP3$^+$ lymphocytes into tumor stroma and within agglomerates of tumor cells were evident (Figure 1*A*). In addition, scattered Foxp3$^+$ lymphocytes were found in the tumor-free lung parenchyma, often in association or even inside blood vessels. Metastatic lymph nodes also contained a large number of Foxp3$^+$ lymphocytes, more prominent around than within tumor metastases itself (Figure 1*B*).

CD4$^+$CD25$^+$Foxp3$^+$ Tregs Accumulate in Lung Tumors

To measure the proportion of lymphocytes infiltrating tumors, we used flow cytometry. We found that the proportion of total CD4$^+$ TIL among CD45$^+$ leukocytes in the center of adenocarcinomas was significantly enhanced, when compared with the tumor border (Figure 2, $p < 0.02$), whereas squamous cell carcinoma samples showed only a nonsignificant difference between the different lung tissue samples. There were no changes in total CD4$^+$ T-cell numbers between healthy lung samples and tumor-infiltrated lung tissue. Similarly, we observed no differences between total CD8$^+$ T cell amounts among CD45$^+$ leukocytes in tumors and in adjacent healthy lung tissues (data not shown).

We found a significantly enhanced proportion of CD4$^+$CD25$^+$Foxp3$^+$ Tregs in the tumor center, when compared with healthy lung tissue and the tumor border (Figure 3*B*; $p < 0.05$ for both). Analyzing tumor samples of different histology, we found that these differences held true for adenocarcinoma (Figure 3*C*; $p < 0.01$), whereas in squamous cell carcinoma, a statistically significant increase was found only in comparison with healthy lung tissue (Figure 3*D*; $p < 0.002$).

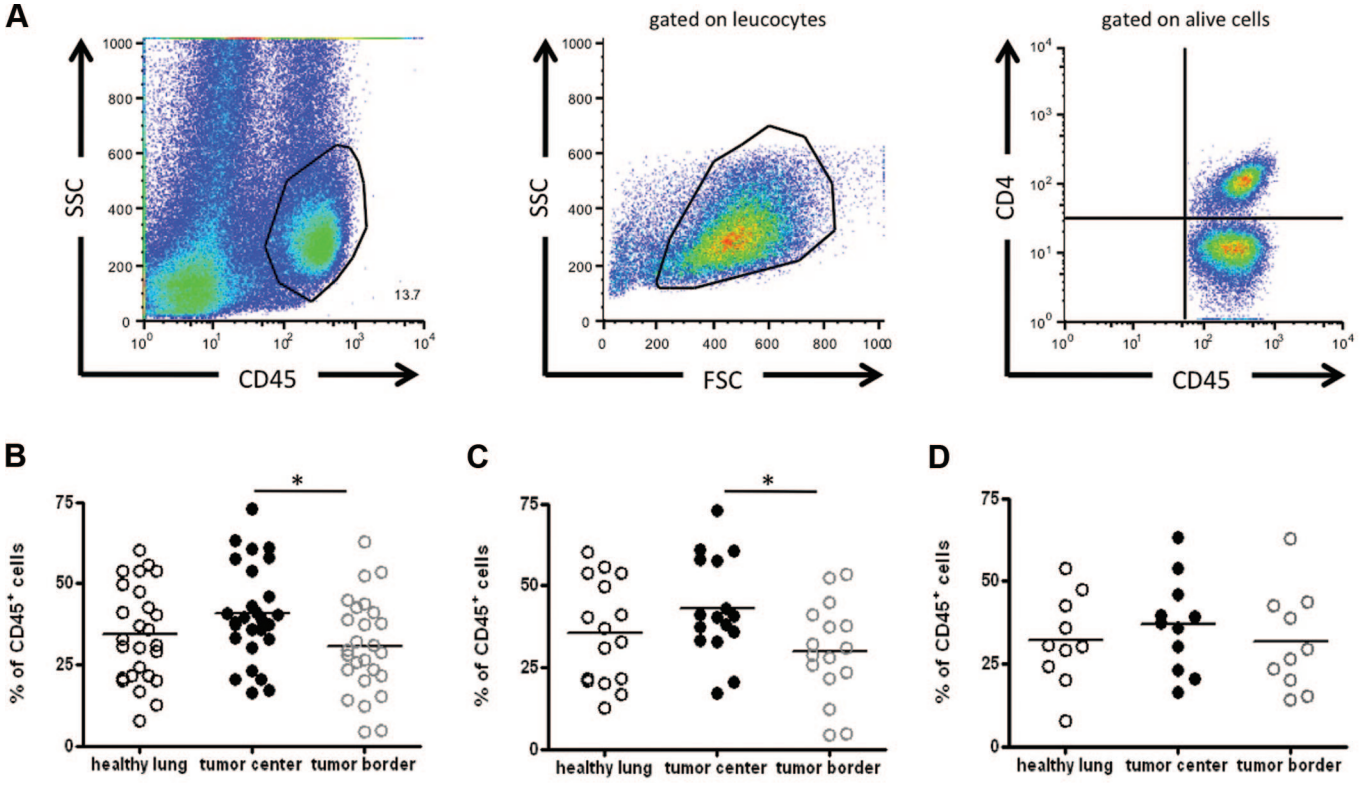

FIGURE 2. Analysis of CD4$^+$ T cells in lung tissue of patients with cancer. Single-cell suspensions prepared from healthy lung tissues, tumor centers, and tumor borders were stained with monoclonal antibodies (mAbs) for CD4 and the leukocyte marker CD45 followed by flow cytometry. *A*, Representative dot plots and gating strategy. *B–D*, Percentage of CD4$^+$ T cells within CD45$^+$ leukocytes in the lung tissue of all patients (*B*; $n = 30$) and of patients with adenocarcinoma (*C*; $n = 16$) or squamous cell carcinoma (*D*; $n = 14$). *$p = 0.02$, significant differences between the groups indicated with the lines.

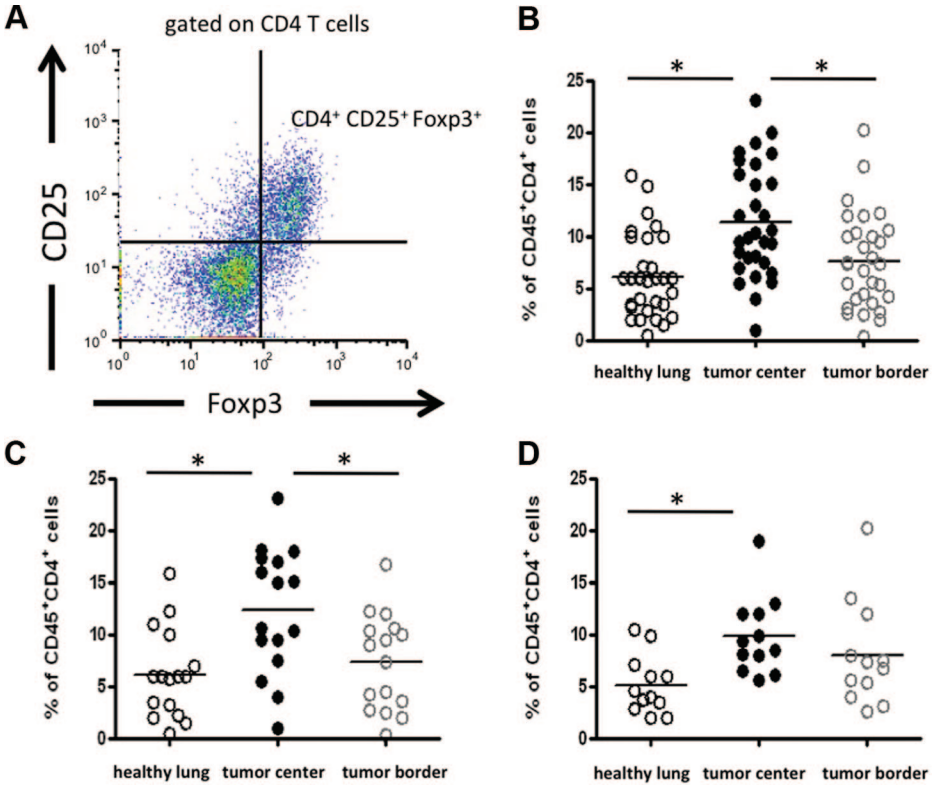

FIGURE 3. Distribution of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) in lung tissue. Cells from healthy lung tissues, tumor centers, and tumor borders were stained with monoclonal antibodies (mAbs) for CD45, CD4, CD25, and FoxP3, followed by flow cytometry. *A,* Representative dot plots and gating strategy. *B–D,* Frequency of $CD4^+CD25^+FoxP3^+$ Tregs in the lung tissue of all patients (*B*; $n = 30$; $*p < 0.05$) and of patients with adenocarcinoma (*C*; $n = 16$; $*p < 0.01$) or squamous cell carcinoma (*D*; $n = 14$; $*p < 0.002$) expressed as percentage within total $CD45^+CD4^+$ T cells.

Interestingly, we found a strong decrease in $CD56^+CD3^-$ NK cells within $CD45^+$ leukocytes in samples from the tumor center, when compared with those from healthy lung tissue and the tumor border (Figure 4*B*; $p < 0.01$). This result was found both to adenocarcinoma and squamous cell carcinoma samples (Figures 4*C*, *D*).

Enrichment of Tregs in Metastatic Lymph Nodes

Flow-cytometric analysis of lymph nodes obtained from patients with adenocarcinoma revealed a significantly enhanced proportion of $CD4^+$ T cells in metastatic, when compared with tumor-free lymph nodes (Figure 5*B*; $p = 0.01$). In squamous cell carcinoma, the enhancement of $CD4^+$ T cells in TIL was less striking and statistically not significant (Figure 5*C*). There was no significant difference in $CD8^+$ and NK cell numbers between metastatic and nonmetastatic lymph nodes (data not shown).

We found significant enhancement of Tregs in metastatic lymph nodes from patients with adenocarcinoma (Figure 6*B*; $p = 0.01$). Nevertheless, in the case of squamous cell carcinoma, the difference between Tregs in metastatic and nonmetastatic lymph nodes was not significant (Figure 6*C*).

Investigating TIL and tumor-free lymph nodes in different positions (local, N1 tumor draining versus distant mediastinal, N2), we observed no significant difference in the number of any studied T-cell subsets, including total $CD4^+$, $Foxp3^+$ Tregs, $CD8^+$, and NK cells.

DISCUSSION

There is growing evidence that Tregs play a key role in suppressing T-cell-mediated immunity in patients with cancer.[6,7] The efficacy of cancer vaccination is enhanced in several animal models by depleting Tregs, whereas an adoptive transfer of Tregs impairs tumor-specific immunity, resulting in tumor progression.[13,14] Increased proportions of Tregs in tumor tissue have been reported in different tumor types, including lung cancer.[15–18] Our findings in tumor-infiltrated lung tissue are in line with a previous report, showing a significantly enhanced proportion of Tregs and fewer NK cells in tumor tissue compared with lung tissue.[9] Interestingly in our series, this difference was also observed between the tumor center and the tumor border. This observation might be explained by an increased concentration of chemokines attracting Treg to the tumor center.[6,7] Importantly, the proportion of NK cells in the center of primary tumors with enhanced Treg infiltration was significantly decreased, when compared with both the healthy tissue and the tumor border. NK cells are considered to be of vital importance to protect from tumor development by their ability to recognize and eliminate transformed cells without previous priming.[19] This elimination depends on the balance between inhibitory and activating signals engaged by ligands expressed on tumor cells.[20] Nevertheless, recent evidence has suggested that Tregs may suppress NK cell effector functions, thereby modulating the reactivity of innate immunity.[21]

In this study, we provided the first evidence of an accumulation of $Foxp3^+$ Tregs in TIL in human NSCLC. In adeno-

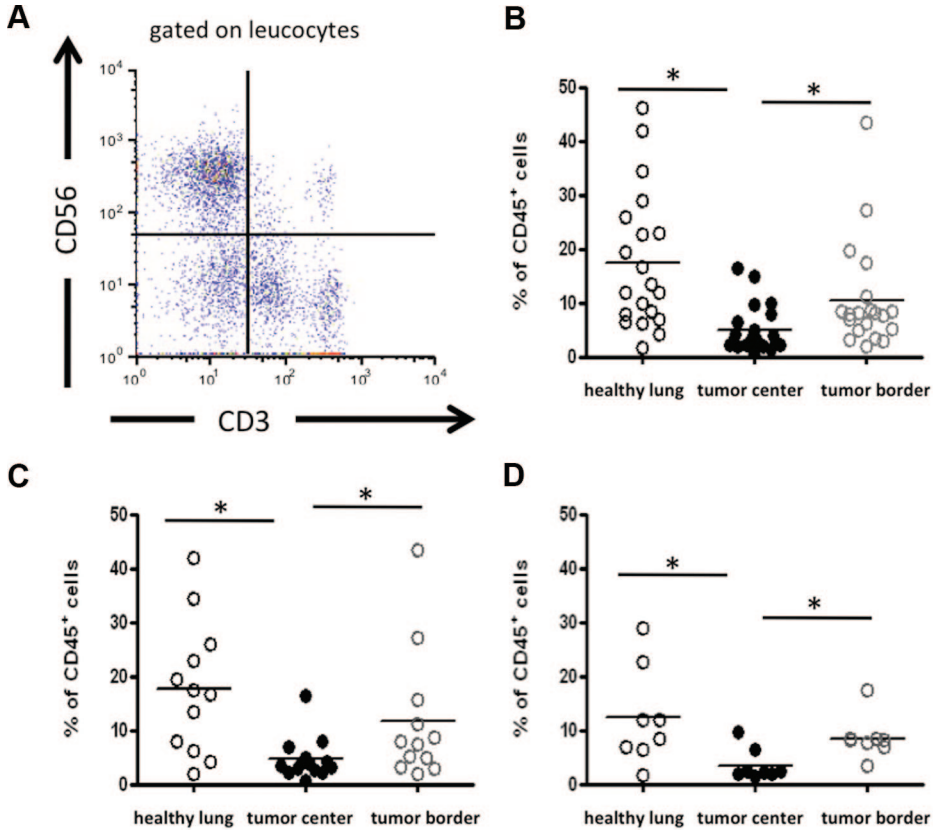

FIGURE 4. Phenotypic analysis of $CD56^+CD3^-$ natural killer (NK) cells in lung tissue. Cells isolated from healthy lung tissues, tumor centers, and tumor borders were stained with monoclonal antibodies (mAbs) for CD45, CD3, and CD56, followed by FACS analysis. *A*, Representative dot plots and gating strategy. *B–D*, Amount of $CD56^+CD3^-$ NK cells in the lung tissue of all patients (*B*; $n = 30$; $*p < 0.01$) and of patients with adenocarcinoma (*C*; $n = 16$; $*p < 0.005$) or squamous cell carcinoma (*D*; $n = 14$; $*p < 0.02$) expressed as percentages of all $CD45^+$ leukocytes.

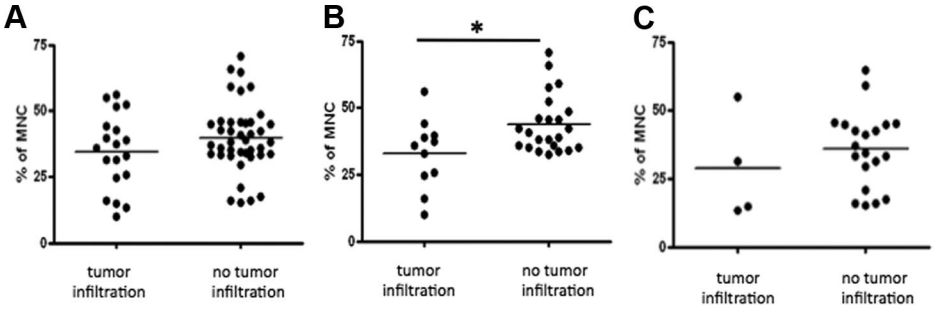

FIGURE 5. Evaluation of $CD4^+$ T cells in lymph nodes from patients with lung cancer. Cell suspensions prepared from tumor-infiltrated lymph nodes, and lymph nodes lacking tumor infiltration were stained with monoclonal antibodies (mAbs) for CD3 and CD4, followed by flow cytometry. *A–C*, Percentage of $CD3^+CD4^+$ T cells within mononuclear cells (MNC) in lymph nodes of all patients (*A*; $n = 30$), of patients with adenocarcinoma (*B*; $n = 16$), or squamous cell carcinoma (*C*; $n = 14$). $*p = 0.01$, significant differences between indicated groups.

carcinomas, the proportion of these cells was significantly enhanced in metastatic lymph nodes compared with lymph node samples without tumor infiltration. Only a few studies have focused on Tregs in tumor-draining lymph nodes in human cancer up to now.[22–24] Similar to our data, increased Treg numbers in tumor-infiltrated draining lymph nodes were reported from patients with gastric cancer.[22] In metastatic melanoma lymph nodes, Tregs were shown to be a major component of the immunosuppressive microenvironment,[25] and recent findings indicate that the immunologic status of sentinel lymph nodes is inhibited before further metastases develop.[26] In contrast to some other tumor types, a typical sentinel lymph node cannot be delineated in lung cancer. Furthermore, the phenomenon of lymph node skipping in the development of lymph node metastases is well known.[27,28] For this reason, lymphoid tissue samples were obtained in this study from the locations closest to the tumor (subsegmental N1 position) and from the most distant (N2) area that is reached by mediastinal lymph node dissection. We found no difference in the studied T-cell subset numbers between samples from local (N1) and mediastinal (N2) lymph nodes. This result was also true when tumor-free N2 lymph nodes obtained from N1-positive patients were compared with those from N1-negative patients. Only tumor infiltration into the lymph nodes resulted in Treg accumulation.

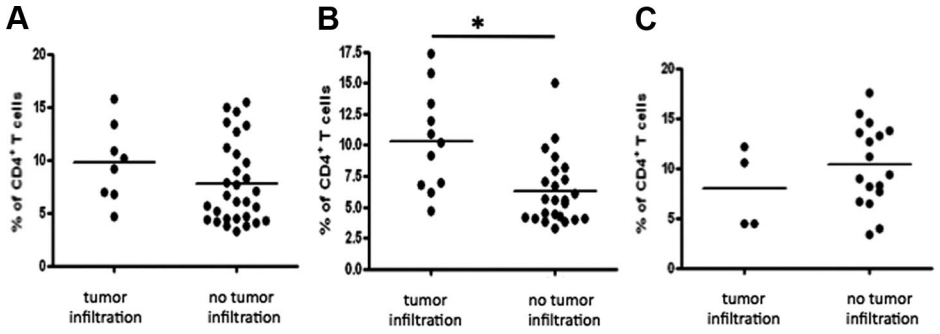

FIGURE 6. Frequency of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) in lymph nodes from patients with lung cancer. Single-cell suspensions prepared from tumor-infiltrated lymph nodes and lymph nodes lacking tumor infiltration were stained with monoclonal antibodies (mAbs) for CD3, CD4, CD25, and FoxP3, followed by FACS analysis. *A–C*, Amount of $CD4^+CD25^+FoxP3^+$ Tregs in the lung tissue of all patients (*A*; $n = 20$) and of patients with adenocarcinoma (*B*; $n = 12$) or squamous cell carcinoma (*C*; $n = 8$) expressed as percentages of all $CD45^+CD4^+$ T cells. *$p = 0.01$, significant differences between indicated groups.

The impact of histological subtypes of NSCLC on Treg accumulation has not been reported up to now. Our findings indicate that adenocarcinoma can create a more profound immunosuppressive microenvironment than squamous cell carcinoma. This observation needs to be confirmed by subsequent studies. Interestingly, similar numbers of Tregs have previously been found studying NSCLC and small cell lung cancer samples.[19] In NSCLC samples, a higher expression of Foxp3 was reported to correlate with decreasing tumor diameter.[29]

A high density of tumor-infiltrating Tregs detected by immunohistochemistry was reported to be associated with the recurrence of resected NSCLC.[30] To correlate numbers of lymphocyte populations with survival was not aim of this study as patient numbers are too small; such a trial using multitissue array analysis is currently ongoing.

In summary, we found an enrichment of immunosuppressive Tregs in primary tumor and tumor-infiltrated lymphoid tissue specimens obtained from patients who received curative surgery for NSCLC in comparison with healthy lung. This observation was particularly enhanced in patients with adenocarcinomas. Treg accumulation was correlated with reduction in NK cell numbers in primary tumors. As NK cells have been demonstrated to play a key role in the antitumor resistance in lungs,[19] its down-regulation associated with Treg expansion in lung tumor tissue may contribute to immunosuppression and tumor progression in patients with lung cancer. Therefore, specific Treg elimination or suppression of their activity may be a therapeutic venue to develop more effective strategies of lung cancer immunotherapy.

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