Tumor infiltrating T cells influence prognosis in stage I–III non-small cell lung cancer

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Background: T cell infiltration in non-small cell lung cancer (NSCLC) is essential for the immunological response to malignant tissue, especially in the era of immune-checkpoint inhibition. To investigate the prognostic impact of CD4⁺ T helper cells (T₉), CD8⁺ cytotoxic (T₈) and FOXP3⁺ regulatory T (T₉ₑ) cells in NSCLC, we performed this analysis.

Methods: By counterstaining of CD4, CD8 and FOXP3 we used immunohistochemistry on tissue microarrays (TMA) to evaluate peritumoral T₉ cells, T₉ₑ cells and T₈ cells in n=294 NSCLC patients with pTNM stage I–III disease.

Results: Strong CD4⁺ infiltration was associated with higher tumor stages and lymphonodal spread. However, strong CD4⁺ infiltration yielded improved overall survival (OS) (P=0.014) in adenocarcinoma (ADC) and large cell carcinoma (LCC) but not in squamous cell carcinoma (SCC). A CD4/CD8 ratio <1 was associated with high grade NSCLC tumors (P=0.020). High CD8⁺ T cell infiltration was an independent prognostic factor for OS (P=0.040) and progression-free survival (PFS) (P=0.012) in the entire study collective. The OS benefit of high CD8⁺ infiltration was especially prominent in PD-L1 negative NSCLC (P=0.001) but not in PD-L1 positive tissue (P=0.335). Moreover, positive FOXP3⁺ expression in tumor infiltrating lymphocytes was associated with increased OS (P=0.007) and PFS (P=0.014) in SCC but not in ADC and LCC (all P>0.05). Here, prognostic effects were prominent in PD-L1 positive SCC (P=0.023) but not in PD-L1 negative SCC (P=0.236).

Conclusions: High proportion of CD8⁺ T₈ cells correlated with improved prognostic outcome in stage I–III NSCLC. T₉ cells and T₉ₑ cells have implications on outcome with respect to tumor histology and biology.

Keywords: Non-small cell lung cancer (NSCLC); tumor infiltrating leukocytes (TILs); CD4; CD8; FOXP3

Submitted Oct 21, 2019. Accepted for publication Apr 22, 2020.

doi: 10.21037/jtd-19-3414a

View this article at: http://dx.doi.org/10.21037/jtd-19-3414a
Introduction

Worldwide, lung cancer harbors paramount position in cancer specific incidence and still is the leading cause of cancer related mortality (1). Despite awareness campaigns, relative incidence ratios of lung cancer in young women resident in western countries are rising (2). With respect to histology, two major subtypes must be distinguished. Whereas non-small cell lung cancer (NSCLC) comprises about 85% of all new-diagnosed cases, small cell lung cancer (SCLC) is the less common (~14%) but more aggressive subtype (3). By genotyping (4) and sequencing oncogene and tumor suppressor gene loci (5), the latter entity of SCLC is nowadays assorted with large cell neuroendocrine carcinomas (LCNEC), typical carcinoids and atypical carcinoids (5-7). Albeit the reclassification of large cell carcinoma (LCC), two distinct histopathological entities represent classical NSCLC subtypes (8,9), i.e., adenocarcinoma (ADC) of the lung and squamous cell carcinoma (SCC) of the lung.

The arise of immunotherapies (10) brought forth a better understanding of lung cancer tumor infiltrating leukocytes (TILs) (11-13). Focusing on therapeutic implications, leukocyte levels and activation markers in lung cancer patients’ blood (14,15) have been investigated. In addition to invading macrophages (12,13), tumor infiltrating T cells are the focus of current research efforts (16,17).

T cells are lymphatic cells that are primarily characterized by expression of the T cell receptor (TCR) and cluster of differentiation 3 (CD3), the T cell co-receptor. As a result of the interaction of the TCR complex with the major histocompatibility complex II (MHC II) of antigen presenting cells, an avalanche maturation and activation process from naïve T cells to effector or memory T cells is initiated. Among T cells, three major subtypes must be distinguished: besides cytotoxic CD3⁺ CD8⁺ T cells (T Servlet), there are two types of CD3⁺ CD4⁺ T cells (18). Polypotent CD4⁺ T cells develop into regulatory CD4⁺ CD25⁺ T cells (Treg) and into conventional CD4⁺ T helper (Th) cells, containing three subtypes (Th1, Th2 and Th17) (19,20). While mature Th1 cells activate macrophages and CD8⁺ T cells via interferon γ (IFNγ) and thereby direct immune response against intracellular pathogens and autoimmunity, Th2 cells mediate defense mechanisms against extracellular pathogens such as helminths by activating eosinophils, basophils and humoral responding B cells via interleukin 4 (IL4) (18). Finally, Th17 cells maintain the immune response against extracellular fungal or bacterial infections by activation of neutrophils via IL17 (20).

With regard to CD4⁺ and CD8⁺ T cells, a higher T lymphocytic infiltration in NSCLC is considered a favorable prognostic marker (13,16,17,21). Studies focused on the interaction of distinct T cell infiltration with epithelial to mesenchymal transition (EMT) in NSCLC: a more ‘mesenchymal state’, predicted by a sixteen-gene signature, was associated with few CD4⁺ cells in ADC and reduced CD4⁺ and CD8⁺ cell counts in SCC (22).

Other than CD4⁺ T Servlet cells, CD25⁺ co-expressing Treg cells occur within peripheral blood, healthy tissues and malignant tissues. Most relevantly, these cells express Forkhead box transcription factor (FOXP3) (23). Of interest, FOXP3 is not exclusively expressed on Treg cells but might also be transiently found on activated CD4⁺ CD25⁺ Th Servlet cells (24,25) and CD8⁺ Th Servlet cells (26). A closer characterization of Treg cells allows the division into suppressive Treg cells, expressing the cytokotic T lymphocyte associated protein 4 (CTLA4), and resting Treg cells that lack CTLA4 (19,23). Treg cells control self-tolerance via cell-cell-interaction and, amongst others, secretion of transforming growth factor β (TGFβ). FOXP3 supports the Treg cell function by mediating downregulation of both T cell expansion and inflammatory cytokine overexpression (27). Hence, high levels of Treg cells proved beneficial in controlling autoimmune disorders whereas depletion of Treg cells is considered to enhance immune response against tumor tissue (20). However, TGFβ itself was found to be a driver of EMT (28), a process that promotes malignancy and metastatic conversion by tumoral loss of cell-cell-junctions and a gain in motility (29). In autoimmune diseases, the suppressive function of thymic Treg cells and circulating Treg cells is well characterized, whereas the role of a Treg cell phenotype detected in tumor microenvironment is ambiguous. Here, tumor infiltrating Treg cells might be substantially different from circulating Treg cells (23). There is evidence that tumor Treg cells share similarity of surface stimulatory and inhibitory receptors with cytotoxic effector T cells (23). Using single cell analysis, De Simone et al. revealed that NSCLC tumoral Treg cells exhibited a tumor specific surface signature, including overexpression of e.g., non-tumoral PD-L1. Here, Treg surface expression levels of PD-L1 were significantly lower when taken from both blood and healthy lung tissue (30). Subsequent to tumor endothelial VEGF secretion, Treg cells migrate into tumor tissue (31,32). Here they ought to orchestrate the immune response by inhibiting antigen presenting cells and cytotoxic T cells via secretion of IL10 and TGFβ as well as by withdrawal of IL2 (27).
Both in colorectal cancer (32) and in head and neck SCC (33), high infiltration rates of T\textsubscript{reg} cells were associated with improved prognosis. In hepatocellular carcinoma however, the effect was vice versa (32). In NSCLC, T\textsubscript{reg} infiltration has been analyzed and discussed very controversially (13). While Kayser \textit{et al.} demonstrated that high levels of CD4\textsuperscript{+} CD25\textsuperscript{+} T\textsubscript{reg} cells were associated with improved survival in patients suffering from ADC and SCC of all stages (34), Kinoshita \textit{et al.} reported unfavorable outcomes in stage I ADC patients when diagnosed high levels of FOXP3\textsuperscript{+} T\textsubscript{reg} infiltration (35). Moreover, stage IV epidermal growth factor receptor (EGFR) mutant ADC patients, pretreated with EGFR-monoconal antibody and nivolumab, benefited of high CD4\textsuperscript{+} and FOXP3\textsuperscript{+} T cellular infiltration (36). In contrast, other studies reported on high FOXP3\textsuperscript{+} T cell infiltration to predict poor survival in NSCLC (37-39).

Taking into account the contrary results of FOXP3 positivity in activated CD4\textsuperscript{+} CD25\textsuperscript{+} T\textsubscript{h} and CD8\textsuperscript{+} T\textsubscript{c} cells (26,40,41), the influence of FOXP3\textsuperscript{+} T\textsubscript{reg} cells in lung cancer progression has not fully been clarified. Cofactors, such as tumor stage, histology and distinct tumor biology might influence the regulatory effect. To investigate the prognostic impact of T\textsubscript{h} cells, T\textsubscript{c} cells and T\textsubscript{reg} cells in stage I–III NSCLC, we performed the underlying, retrospective immunohistochemical analysis with focus on specific markers such as CD4, CD8 and FOXP3.

**Methods**

**Study collective**

Retrospectively, we analyzed n=294 cases of NSCLC patients, who were surgically treated at the Thoracic Surgery Department of the St. Georg’s Clinic Ostercappeln between December 1998 and November 2004. In consequence of the therapeutic dates, the sixth edition of tumor nodule metastasis (TNM) system proposed by the Union Internationale Contre le Cancer (UICC) (42) was applied. An update towards later TNM staging system editions was not possible due to ethical concerns regarding privacy and data protection.

**Tissue preparation**

We analyzed the surgically resected primary tumor tissue samples by using 4-µm-thick formalin-fixed paraffin-embedded (FFPE) tissue microarrays (TMA). Three punch cores from the original tumor specimen solely represented one single NSCLC case in the present cohort (43). Tissue preparation was performed according to the suggestions of von Wasielewski \textit{et al.} (44). In detail, the hematoxylin and eosin stained primary tumor slides were used to identify representative and non-necrotic tumor plots including both, vital tumor cells as well as stromal cells and infiltrates. Moreover, three punch cores ought to dissociate at least 1 mm within the primary tumor block. These areas were then marked on the paraffin embedded tumor block and punched out with a 0.6 mm biopsy needle. Afterwards, gained tissue was melted within in the accepting paraffin block.

**Immunohistochemistry**

We used the peroxidase-conjugated avidin-biotin method to perform immunohistochemistry. Antibodies included rabbit CD4 monoclonal primary antibody (Roche/Ventana clone SP35; Catalog Number 790-4423), rabbit CD8 monoclonal primary antibody (Roche/Ventana clone SP57; Catalog Number 790-4460) and rabbit FOXP3 polyclonal primary antibody (Abcam clone ab4728; Catalog Number ab4728). In sum, TMA sections were deparaffinized in xylene and rehydrated through graduated ethanol-solutions at ambient temperature. We incubated the primary antibodies for 30 minutes at room temperature. After washing, the incubation of the paraffin sections with biotinylated secondary antibodies was performed. Using 3-amino-9-ethylcarbazole as a substrate, immunoreactions were detected (Roche/Ventana Optiview DAB IHC detection KIT; Catalog Number 760-700). Positive controls were performed on human tonsil tissues (Figure S1).

**Tissue analysis**

Tissue was analyzed at a Leica DM4B and an Olympus BX51 microscope with an average magnification of ×200 by ABS, LHS and BH. Deviations in the microscopic analysis were discussed interdisciplinary. To detect distinct staining in FOXP3 stained tissue, an intermittent magnification of ×400 was allowed. The TIL infiltration grade was studied in FOXP3 stained tissue, an intermittent magnification of ×400 was allowed. The TIL infiltration grade was studied in accordance to the method proposed by the International TILs Working Group 2014 (45). Hence, TIL infiltration was documented as percent of the cells on one punch core. Subsequently, CD4\textsuperscript{+}, CD8\textsuperscript{+} and FOXP3\textsuperscript{+} cells were then assessed as percent of TILs. As proposed by the International TILs Working Group 2014, the analyzed TILs derived from stromal areas and excluded granulocytes and macrophages in necrotic or inflammatory sections (45).
Additionally, highly infiltrated areas by granulocytes or macrophages were visually excluded from the analysis. As each NSCLC case was represented by three punch cores, each evaluated case accommodated a maximum of three independent TIL counts and three independent counts of CD4⁺, CD8⁺ and FOXP3⁺ cells, respectively. Data was later on harmonized to a mean and median TIL count as well as mean and median CD4⁺, CD8⁺ and FOXP3⁺ cell count, each dependent and independent from TIL infiltration grade.

**Post-hoc PD-L1 correlations**

With regard to Schmidt et al. (46), PD-L1 stains were performed on this cohort beforehand via rabbit PD-L1 monoclonal primary antibody (Cell Signaling Technology clone E1L3N, Catalog Number 13684). Herein, n=293 cases could be matched to perform post-hoc correlative analyses of PD-L1 and TIL infiltration. As described by Schmidt et al., tumors were evaluated PD-L1 positive, if at least 5% of tumoral tissue was stained positively. Moreover, staining intensity was semi-quantitatively documented via the immunoreactive score (IRS) (i.e., 0= no staining, 1= mild staining, 2= moderate staining, 3= intense staining) (47). To simplify analysis, IRS 0–1 tissue was deduced ‘PD-L1 negative’ and IRS 2–3 tissue ‘PD-L1 positive’.

**Statistical analysis**

Mean, standard deviation (SD), median, interquartile range (IQR; Q1–Q3) as well as raw count and frequencies were used to describe the patient cohort. Twofold associations between categorical variables were analyzed via Fisher’s exact test or Chi-square test, if applicable. Continuous and ordinal variables were tested using either unpaired t-test or Mann-Whitney-U test depending on the normality of the data. Associations of more than two groups (e.g., stage variable with categorical and continuous outcomes were analyzed using Chi-square test, Kruskal-Wallis test or via one-way-ANOVA, in case of continuous and normally distributed values. The area under the receiver operating characteristic curve (ROC) was used to approximate cutoff values for CD4⁺ and CD8⁺ cells via Youden Index for binary classification split by median overall survival (OS).

OS was defined as time (days) between histopathological diagnosis and death or censoring. Progression-free survival (PFS) was defined as time (days) between histopathological diagnosis and first relapse, progress after initial treatment, death or censoring, depending on the first chronological appearance. Univariate survival analyses compared OS and PFS between groups by using log-rank tests. Kaplan-Meier plots then were used to visualize survival differences. Multivariate survival analyses were performed via Cox proportional hazards model by applying likelihood ratio tests with a forward variable selection. Inclusion criterion was set to 0.05. Hazard ratios (HR) are presented with 95% confidence intervals (95% CI). Data collection as well as calculations were performed using IBM® SPSS® Statistics Version 25 (released 2017, IBM Corp., Armonk, NY, USA). Graphic data output was partly generated in Microsoft Office Professional Plus 2010 Excel (released 2010, Microsoft Corp., Redmond, WA, USA). The local significance level was set to 0.05. Due to the explorative character of the analysis, an adjustment to multiplicity was not determined.

**Results**

**Baseline characteristics**

Baseline data of the investigated study cohort of n=294 NSCLC patients with stage I–III disease is summarized in Table 1. Briefly, 80% of the patients were male. Mean age at time of diagnosis was 65.3 years. While 93% of the patients had a good performance (ECOG 0–1), 7% revealed limited performance status (ECOG II–III) previous to NSCLC diagnosis. 77% of the patients were smokers and the mean relative forced expiratory volume in 1 second (FEV1) was 80.8% (±20.1%). Regarding tumor histology, 48% exhibited SCC morphology, 38% were ADC and 14% LCC. Furthermore, one third of the tumors had a low tumor grade (G1 and G2), whilst two thirds had high tumor grades (G3 and G4). Following the UICC 6th edition TNM proposal, 53% of the patients had stage I disease, 25% had stage II tumors and 22% stage III tumors. More specifically, lymphonodal spread (pN+) was found in 39% of the cases. While 70% of the patients did not receive any neoadjuvant treatment, 30% were treated by radiotherapy or chemotherapy before surgical treatment and acquisition of tumor specimen. PD-L1 stain was negative in three fourth of the specimen (IRS 0–1). The mean TIL count was 26.7%. Of these, mean and median amounts of CD4⁺ cells were 22% and 20%, of CD8⁺ cells 24% and 20% and of FOXP3⁺ cells 2% and 0%, respectively. In terms of survival, median PFS was 932 [95% confidence interval (CI), 691–1,173] days, median OS was 1,316 (95% CI,
986–1,646) days, and median follow up was 2,686 (95% CI, 2,557–2,815) days, respectively.

**TIL associations with histomorphological and clinicopathological features**

Figure 1 exemplary depicts the implemented CD4 (Figure 1A,C,E) and CD8 (Figure 1B,D,F) stains on matching cores. Whereas Figure 1A,B represent lymphocytic stains in stroma of a well differentiated tumor, Figure 1C,D reveal a predominant CD4+ TIL infiltration on a stromal column, whereas CD8+ TILs can be found in tumor area as well as in the column of stroma. Figure 1G,H depict negative and positive FOXP3 TIL stains.

Figure 2 reveals associations of TIL-infiltration in general and CD4+, CD8+ and FOXP3+ T lymphocytic infiltration with tumor-biologic markers such as histology, lymphonodal spread, TNM-stage and tumor grading. Interestingly, FOXP3+ T cells appeared less frequent in ADC (mean infiltration 1.03%) than in SCC (2.35%) or LCC (2.34%) (P=0.006). No significant difference between morphologic entities ADC, SCC and LCC was found for percentage TIL count, CD4+ or CD8+ count (Figure 2A). In terms of CD4+/CD8 ratio, CD4 dominance (ratio >1) was associated with low grade tumors, and CD8 dominance (ratio <1) with high grade tumors (P=0.020) (Figure 2D). While the pT stage 1–4 was not associated with higher levels of TILs, CD4+ T cells, CD8+ T cells or FOXP3+ T cells (all P>0.05, data not shown), CD4+ cells were more present in pN2 tumors (28.0% CD4+ TILs) than in pN0 (20.5%) or pN1 (20.8%) cases (P=0.014). No such associations were found for CD8 or FOXP3, respectively (all P>0.05) (Figure 2B). As a consequence of the pN stage, there was an increase in CD4 levels in TILs at higher tumor stages (stage I 19.8%, stage II 22.6%, stage III 26.2%, P=0.034). Here, FOXP3 density was associated with stage II tumors (P=0.036), too (Figure 2C). Associations of TIL count, CD4+ TIL count, CD8+ TIL count or FOXP3+ TIL count were neither found for age (<65, ≥65 years), sex (male, female), ECOG (0, 1, 2, 3), smoking status (non-smoker, smoker) nor neoadjuvant treatment (no neoadjuvant treatment, neoadjuvant treatment) (all P>0.05).

**Classification and characteristics of CD4+, CD8+ and FOXP3+ expression split subcohorts**

In terms of prognostic impact of CD4+ and CD8+ T lymphocyte infiltration in tumor tissue, we performed an area under the receiver operating characteristic curve analysis via Youden index for median OS. Highest sensitivity and specificity was bordering 20% cutoff value for CD4+ and CD8+ cells, respectively. Hence, we compared low infiltration rates (i.e., <20% of TILs) with high infiltration rates (i.e., ≥20% of TILs). For FOXP3+ cells, we compared specimen absent of FOXP3+ T_reg cells (i.e., 0% of TILs) with FOXP3+ detected cells in tumor and stromal tissue (i.e., ≥1% of TILs). Subsequently, the deriving subcohorts were analyzed; see Table S1 and Figure 2. Here, n=161 patients revealed low CD4+ infiltration rates, whereas n=133 patients had a high CD4+ lymphocytic infiltration. With respect to age, gender, smoking behavior, histopathology, tumor grading and pT stage, no significant difference in high and low CD4+ infiltrated tumor tissue specimen was detected (all P>0.05). In line with the raw TIL counts, high CD4+ lymphocyte infiltration predicted pN2 tumors (P=0.039) and higher pTNM stages (P=0.047), see also Figure 2B,C. Neither for high (n=155) or low (n=139) CD8+ infiltrated tumors, nor for tumors with (n=123) or without FOXP3+ infiltrating cells (n=171) differences in age, gender distribution, smoking status, histopathology, tumor grading, pT, pN or pTNM staging any differences in the derived subcohort were found (all P>0.05). As a consequence of the proposed subcohorts for CD4+ and CD8+ dichotomy, mean TIL count was significantly lower in the low CD4+ (P=0.002) and low CD8+ subgroup (P<0.001), see Table S1.

**Univariate OS and PFS analysis**

Kaplan-Meier curves of univariate survival analyses regarding CD4+, CD8+ and FOXP3+ TIL infiltration can be found in Figure 3. Univariate survival analyses yielded improved prognostic outcome of tissue with high CD8+ TIL levels (P=0.01 for OS, P=0.015 for PFS, Figure 3C,D). Neither the presence of FOXP3+ cells (Figure 3E,F) nor high CD4+ infiltration levels (Figure 3A,B) resulted in improved OS or PFS, univariately (all P>0.05). However, when focusing on SCC patients solely, FOXP3+ T_reg cells were associated with improved PFS (P=0.027) and OS (P=0.018) (Figure 4A,B). However, in ADC and LCC no association of FOXP3+ T_reg cells with survival was found (Figure 4C,D,E,F). Despite specific T cell subsets, TIL density itself did not affect PFS or OS (all P>0.05, data not shown).

**Multivariate OS and PFS analysis**

Multivariate prognostic analyses for OS and PFS were
Figure 1 Stains for CD4, CD8 (A,B,C,D,E,F) and FOXP3 (G,H). Representative Cores of CD4⁺ and CD8⁺ TILs on TMA tissue specimen #40 (A: CD4, B: CD8), #195 (C: CD4, D: CD8) and #31 (E: CD4, F: CD8), respectively. Negative FOXP3 stain on tissue specimen #40 (G) and positive stromal T<sub>reg</sub> FOXP3 stain on tissue specimen #193 (H). Photographs taken on an Olympus BX51 microscope at ×100 magnification. Detailed views at ×400 magnification can be found in the upper or lower right corner. White squares indicate the area of magnification. TILs, tumor infiltrating leukocytes; TMA, tissue microarrays; T<sub>reg</sub>, regulatory T.
performed for the whole cohort (n=294, Table 2) as well as for SCC (n=140, Table 3) and ADC plus LCC subcohorts (n=154, Table 4), respectively. While analyzing the whole cohort, we used histologic growth pattern (SCC vs. ADC vs. LCC) as an additional independent factor for the analysis. By multivariate survival analysis of the whole cohort as well as of SCC and ADC plus LCC subgroups we evaluated the independent factors of age (≤65 vs. ≥65 years), sex (female vs. male), UICC pTNM 6th edition stage (stage I vs. stage II vs. stage III), tumor grading (G1/2 vs. G3/4), CD4+ (≥20% TILs vs. <20% TILs), CD8+ (≥20% TILs vs. <20% TILs) and FOXP3+ (≥1% TILs vs. 0% TILs). In the full cohort (Table 2), next to age, histology and TNM stage, a low CD4+ TIL infiltration (<20% of TILs: HR: 1.50, P=0.05) and higher CD8+ infiltration (≥20% of TILs: HR: 1.36, P=0.04) were predictors of dismal OS. For PFS, only histology, TNM stage and CD8+ infiltration were independent prognostic factors, respectively. However, focusing on the SCC subgroup (Table 3), apart from the TNM stage, only low CD8+ TIL infiltration (HR: 1.52 for OS, HR: 1.53 for PFS, all P<0.05) and negative FOXP3+ Treg infiltration (HR: 1.81 for OS, HR: 1.74 for PFS, all P<0.02) were independent predictors of dismal outcome. Yet, in ADC and LCC subgroup (Table 4), only CD4+ infiltration (<20% of TILs: HR: 1.80, P<0.02) sustained an independent prognostic factor for OS, along with age, sex and TNM stage.

Post-hoc PD-L1 correlations and survival analysis

We performed post-hoc PD-L1 tumor stain correlations (46) with CD4+, CD8+ and FOXP3+ tumor infiltrating lymphocytes. Raw and percentage counts of the evaluations can be found in Table S2. The infiltration ratio of CD4+ lymphocytes was not associated with PD-L1 negativity or positivity, irrespective of the tumors histology.
Table S2

|                | Pos | Neg |
|----------------|-----|-----|
|                |     | 365 |
|                | 123 | 171 |
|                | 81  | 87  |
|                | 54  | 50  |
|                | 23  | 26  |
|                | 5   | 7   |
|                | 0   | 0   |
|                | 0   | 0   |
|                |     | 365 |
|                | 123 | 171 |
|                | 81  | 87  |
|                | 54  | 50  |
|                | 23  | 26  |
|                | 5   | 7   |
|                | 0   | 0   |
|                | 0   | 0   |
|                |     | 365 |
|                | 123 | 171 |
|                | 81  | 87  |
|                | 54  | 50  |
|                | 23  | 26  |
|                | 5   | 7   |
|                | 0   | 0   |
|                | 0   | 0   |

Figure 3 OS and PFS depending on CD4+, CD8+ and FOXP3+ TIL infiltration. The infiltration density of CD4+ cells in TILs neither influences OS (A) nor PFS (B) of NSCLC stage I–III patients; a high CD8+ cell proportion in TILs favors OS (C) and PFS (D) of the analyzed patients; FOXP3+ cell proportion of TILs does not alter OS (E) or PFS (F) in the overall cohort. OS, overall survival; PFS, progression-free survival; TIL, tumor infiltrating leukocyte; NSCLC, non-small cell lung cancer.

(SCC P=0.835, ADC P=1.000, LCC P=0.734, data not shown). Interestingly, high CD8+ infiltration was correlated with PD-L1 positive tumors (P=0.001). Likewise, the presence of FOXP3+ Treg cells was significantly more likely in PD-L1 positive NSCLC tissues (P<0.001), irrespective of the underlying histology (SCC P=0.006, ADC P=0.023, LCC P=0.002, see Table S2). Univariate OS analyses split by low and high CD8+ lymphocytic infiltration resulted in beneficial survival for high CD8+ TILs only in PD-L1 low tissue (i.e., IRS 0–1, P=0.001) but not in PD-L1 high tissue (i.e., IRS 2–3, P=0.335) in the overall cohort. On the contrary, in SCC, FOXP3+ Treg cells now resulted in improved survival only in PD-L1 overexpressing tissue (i.e., IRS 2–3, P=0.023) but not in PD-L1 low tumors (i.e., IRS 0–1, P=0.263). Corresponding Kaplan-Meier curves can be found in the Figure S2.

**Discussion**

Among lymphocytic infiltration in NSCLC tissues, T cells
### Table 1 Baseline characteristics of the cohort

| Baseline characteristics | n (total) =294 | % of non-missing values |
|--------------------------|---------------|-------------------------|
| Age (years)              |               |                         |
| Mean (± SD)              | 65.3 (±8.6)   |                         |
| Median (Q1–Q3)           | 65.5 (61.4–70.8) |                       |
| Sex                      |               |                         |
| Male                     | 234           | 79.6                    |
| Female                   | 60            | 20.4                    |
| ECOG                     |               |                         |
| 0                        | 44            | 15.0                    |
| I                        | 230           | 78.2                    |
| II–III                   | 20            | 6.8                     |
| Smoking status (n=293)   |               |                         |
| Negative                 | 67            | 22.9                    |
| Positive                 | 226           | 77.1                    |
| FEV1 (rel. %)            |               |                         |
| Mean (± SD)              | 80.8 (±20.1)  |                         |
| Median (Q1–Q3)           | 81 (67.0–96.0) |                       |
| Histopathology           |               |                         |
| SCC                      | 140           | 47.6                    |
| ADC                      | 113           | 38.4                    |
| LCC                      | 41            | 13.9                    |
| Grade                    |               |                         |
| G1                       | 3             | 1.0                     |
| G2                       | 92            | 31.3                    |
| G3                       | 163           | 55.4                    |
| G4                       | 36            | 12.2                    |
| Resection (n=291)        |               |                         |
| R0                       | 276           | 94.8                    |
| R1                       | 13            | 4.5                     |
| R2                       | 2             | 0.7                     |
| UICC6 pT                 |               |                         |
| pT1                      | 95            | 32.3                    |
| pT2                      | 160           | 54.4                    |
| pT3                      | 27            | 9.2                     |
| pT4                      | 12            | 4.1                     |

*Table 1 (continued)*
| Baseline characteristics | n (total) =294 | % of non-missing values |
|--------------------------|----------------|------------------------|
| **UICC6 pN**             |                |                        |
| pN0                      | 178            | 60.5                   |
| pN1                      | 63             | 21.4                   |
| pN2                      | 53             | 18.0                   |
| **UICC6 cM**             |                |                        |
| cM0                      | 294            | 100.0                  |
| **UICC6 pTNM stage**     |                |                        |
| Stage I                  | 155            | 52.7                   |
| Stage II                 | 73             | 24.8                   |
| Stage III                | 66             | 22.4                   |
| **Tumoral PD-L1 stain (n=293)** | | |
| Negative (IRS 0)         | 100            | 34.1                   |
| Mild positive (IRS 1)    | 120            | 41.0                   |
| Moderate positive (IRS 2)| 50             | 17.1                   |
| Intense positive (IRS 3) | 23             | 7.8                    |
| **Neoadjuvant treatment**|                |                        |
| No neoadjuvant treatment | 206            | 70.1                   |
| Chemotherapy/radio-chem  | 88             | 29.9                   |
| **Adjuvant treatment**   |                |                        |
| No adjuvant treatment    | 229            | 77.9                   |
| Chemotherapy             | 13             | 4.4                    |
| Radiotherapy             | 52             | 17.7                   |
| **TIL count (%)**        |                |                        |
| Mean (± SD)              | 26.7 (±13.5)   |                        |
| Median (Q1–Q3)           | 25 (16.7–33.8) |                        |
| **CD4⁺ count (%)**       |                |                        |
| Mean (± SD)              | 6.6 (±7.4)     |                        |
| Median (Q1–Q3)           | 4.4 (1.7–9.7)  |                        |
| **CD4⁺ count (% TIL)**   |                |                        |
| Mean (± SD)              | 21.9 (±17.1)   |                        |
| Median (Q1–Q3)           | 20 (5.0–30.0)  |                        |
| **CD8⁺ count (%)**       |                |                        |
| Mean (± SD)              | 7.1 (±6.6)     |                        |
| Median (Q1–Q3)           | 5.3 (3.0–9.1)  |                        |
dominate (14). With regard to checkpoint inhibition, CD8⁺ T cells in particular are in the scientific focus since anti-tumoral activity of cytotoxic T cells can be interfered by PD-1/PD-L1 axis of tumoral self-tolerance (10). Here, we depicted the influence of T cells, characterized by the expression of CD4, CD8 and FOXP3, in NSCLC tumor microenvironment. Basically, the underlying analysis was carried out to obtain clinicopathologic and prognostic information with focus on T cellular infiltration in NSCLC. However, the presented data derives retrospective analyses. Moreover, we did not adjust p-values to multiple testing. Therefore, the presented statements may only be used to generate or prove hypotheses.

As revealed by flow cytometry in a smaller cohort (14), we support the thesis, that histology does not affect CD4⁺ or CD8⁺ T cell infiltration (Figure 2A). While CD8⁺ T cells were neither associated with primary tumors’ size (pT), nodal status (pN) nor with pTNM stage (Figure 2A,B,C), a higher CD4⁺ TIL density correlated with the event of lymphonodal spread (N2) (Figure 2B) and higher pTNM tumor stages (Figure 2C). In terms of tumor grading, however, the CD4/CD8 ratio was shifted in favor of CD8⁺ for high-grade tumors (G3/4) (Figure 2D). With respect to tumoral PD-L1 stain, we were able to demonstrate, that the beneficial prognostic effect of tumor infiltrating CD8⁺ lymphocytes does not account for tumors with high PD-L1 expression (Figure 2A,B).

EMT is a crucial pathway in cancerogenesis affecting invasiveness of cancer cells by promoting motility and trans-endothelial migration. This process can be studied by analyzing the tumor for ‘mesenchymal’ markers such as N-cadherin (48) and vimentin (49). Both factors are overexpressed in high grade NSCLC (G3/4) indicating that dedifferentiation of the tumor from its cell origin might result from EMT. In this context, our results support the idea, that tumor grading is associated with EMT, since the

| Baseline characteristics | n (total) =294 | % of non-missing values |
|--------------------------|---------------|------------------------|
| CD8⁺ count (% TIL)       |               |                        |
| Mean (± SD)              | 24.1 (±13.7)  |                        |
| Median (Q1–Q3)           | 20 (10.0–30.0)|                        |
| FOXP3⁺ count (%)         |               |                        |
| Mean (± SD)              | 0.6 (±1.3)    |                        |
| Median (Q1–Q3)           | 0 (0.0–0.75)  |                        |
| FOXP3⁺ count (% TIL)     |               |                        |
| Mean (± SD)              | 1.8 (±3.4)    |                        |
| Median (Q1–Q3)           | 0 (0.0–1.0)   |                        |
| PD-1⁺ TIL count (%) (n=292) |            |                        |
| Mean (± SD)              | 3.1 (±8.0)    |                        |
| Median (Q1–Q3)           | 0 (0.0–0.0)   |                        |
| PFS (days)               |               |                        |
| Median (95% CI)          | 932 (690.8–1,173.2) |                    |
| OS (days)                |               |                        |
| Median (95% CI)          | 1,316 (986.4–1,645.6) |                    |
| Follow up (days)         |               |                        |
| Median (95% CI)          | 2,686 (2,557.1–2,814.9) |                    |

SD, standard deviation; FEV1, forced expiratory volume in 1 second; SCC, squamous cell carcinoma; ADC, adenocarcinoma; LCC, large cell carcinoma; IRS, immunoreactive score; TIL, tumor infiltrating leukocyte; PFS, progression-free survival; OS, overall survival; CI, confidence intervals.
orchestration of \( T_h \), \( T_{\text{reg}} \) and \( T_c \) cellular immunosurveillance in NSCLC is directly linked to EMT. Of interest, in ADC decreasing fold change of \( T_{\text{reg}} \) cells in the tumor microenvironment was found in a higher ‘mesenchymal state’ in EMT, while in SCC \( T_{\text{reg}} \) cell depletion has not been described in the process of EMT (22). We were able to reproduce these findings, by documenting reduced FOXP3 infiltration in ADC but not in SCC or LCC, see Figure 2A. Still, the number of T cells in the tumor did not change in EMT, but activated CD4+ \( T_h \) cells were especially prominent in ‘epithelial state’ tumors in both ADC and SCC (22). This correlates with the grade-dependent CD4/CD8 ratio finding in Figure 2D. However, for pancreatic ADC cells (50), IFN\( \gamma \), eventually produced by CD4+ \( T_h \),
cells, was shown to induce EMT and the overexpression of PD-L1 on tumor tissue. And yet in NSCLC, both ADC and SCC presented significantly higher levels of TGFβ1, IL2, IL10 and IFNγ in a ‘mesenchymal state’ compared to the ‘epithelial state’ (22). Alas in this NSCLC cohort, CD4+ lymphocytic infiltration neither deduced to PD-L1 positive SCC, ADC nor LCC (all P>0.05, data not shown).

Interestingly, Vahl et al. detected for ADC that the ‘immunosuppressive’ IL10, inter alia produced by M2 macrophages, activated T<sub>reg</sub> cells and lead to both tumoral and FOXP3<sup>+</sup> T<sub>reg</sub> cellular upregulation of the IL10 receptor (IL10-R). By negative feedback via the T<sub>reg</sub> cells, this finally resulted in less PD-L1 expression in ADC and contributed to a milieu enriched by pro-tumor IL10, as well as the presence of M2 macrophages and PD1<sup>+</sup> T<sub>h</sub> exhausted cells. However, in SCC the presence of IL10 resulted in tumoral but not in T<sub>reg</sub> cell upregulation of IL10-R. Likewise, IL10-R upregulation correlated with higher levels of tumoral PD-L1 (51). Alas, anti-tumoral implications of T<sub>reg</sub> cell presence in SCC were not further elucidated. Unlike Vahl et al., Giatromanolaki et al. and Silva et al. showed, that high FOXP3 infiltration levels were associated with high PD-L1 stain, irrespective of histology (21,52). Our findings enqueue to the positive association of PD-L1 expressing

### Table 3 Cox proportional hazards model for the SCC subcohort

| SCC (n=140) |   |   |   |   |
|-------------|---|---|---|---|
|             | PFS          | OS         |
|             | HR (95% CI) | P value | HR (95% CI) | P value |
| Age         | 0.426        | 0.848     |
| <65 years*  |             |           |             |           |
| ≥65 years   |             |           |             |           |
| Sex         | 0.323        | 0.583     |
| Female*     |             |           |             |           |
| Male        |             |           |             |           |
| UICC 6 TNM stage |   | <0.001 |   | <0.001 |
| I†          |             |           |             |           |
| II          | 2.04 (1.20–3.47) | 0.008 | 1.94 (1.16–3.25) | 0.012 |
| III         | 3.78 (2.26–6.34) | <0.001 | 3.49 (2.10–5.81) | <0.001 |
| Grading     | 0.424        | 0.396     |
| Low grade (G1/G2)* |   |           |   |           |
| High grade (G3/G4) |             |           |             |           |
| CD4<sup>+</sup> | 0.697 | 0.153     |
| High (≥20% TIL)* |             |           |             |           |
| Low (<20% TIL) |             |           |             |           |
| CD8<sup>+</sup> | 0.049 | 0.046     |
| High (≥20% TIL)* |             |           |             |           |
| Low (<20% TIL) |             |           |             |           |
| FOXP3<sup>+</sup> | 0.014 | 0.007     |
| Positive (≥1% TIL)* |             |           |             |           |
| Negative (0% TIL) | 1.74 (1.12–2.71) | 1.81 (1.18–2.77) | |

Cox proportional hazards model using a forward likelihood ratio test; inclusion criterion 0.05; *, index variable; †, reference variable for type III test; italic values are not significant. PFS, progression-free survival; OS, overall survival; CI, confidence intervals; HR, hazard ratios; SCC, squamous cell carcinoma; TIL, tumor infiltrating leukocyte.
Table 4  Cox proportional hazards model for the ADC plus LCC subcohort

| ADC + LCC (n=154) | PFS | OS |
|-------------------|-----|----|
|                   | HR (95% CI) | P value | HR (95% CI) | P value |
| Age               | 0.008 | 0.005 |
| <65 years*        | 1.83 (1.17–2.87) | 1.84 (1.20–2.83) |
| ≥65 years         | 0.039 | 0.045 |
| Sex               | 1.74 (1.03–2.94) | 1.65 (1.01–2.70) |
| Female*           | 1.74 (1.03–2.94) | 1.65 (1.01–2.70) |
| Male              | 0.063 | 0.108 |
| UICC 6 TNM stage  | <0.001 | <0.001 |
| I                 | 2.11 (1.22–3.65) | 2.02 (1.21–3.37) | 0.007 |
| II                | 3.42 (2.04–5.74) | <0.001 | 4.07 (2.33–7.09) | <0.001 |
| III               | 0.065 | 0.062 |
| Grading           | 0.174 | 0.173 |
| Low grade (G1/G2)*| 0.174 | 0.173 |
| High grade (G3/G4)| 0.174 | 0.173 |
| CD4+              | 0.061 | 0.014 |
| High (≥20% TIL)*  | 1.80 (1.13–2.86) | 0.062 |
| Low (<20% TIL)    | 0.065 | 0.062 |
| CD8+              | 0.174 | 0.173 |
| High (≥20% TIL)*  | 1.80 (1.13–2.86) | 0.062 |
| Low (<20% TIL)    | 0.065 | 0.062 |
| FOXP3+            | 0.174 | 0.173 |
| Positive (≥1% TIL)*| 0.174 | 0.173 |
| Negative (0% TIL) | 0.174 | 0.173 |

Cox proportional hazards model using a forward likelihood ratio test; inclusion criterion 0.05; *, index variable; †, reference variable for type III test; italic values are not significant. PFS, progression-free survival; OS, overall survival; CI, confidence intervals; HR, hazard ratios; ADC, adenocarcinoma; LCC, large cell carcinoma; TIL, tumor infiltrating leukocyte.

tumors with a higher prevalence of FOXP3+ T_T cell infiltrates, irrespective of tumor histology (SCC P=0.006, ADC P=0.023, LCC P=0.002).

With respect to prognosis, CD4+ TIL infiltration, prominent in ‘epithelial state’ NSCLC (22), was associated with an improved OS in our multivariate analysis (Table 2). In addition, CD4+ TIL infiltration affected OS in the ADC plus LCC cohort (Table 4), while CD4+ TIL infiltration for SCC had no prognostic impact (Table 3). For SCC, Sterlacci et al. stated a high CD4/CD8 (≥1) ratio to be associated with inferior outcome (53). Alas, we could not confirm these findings in our study cohort (Figure S3). Next, we identified strong CD8+ TIL infiltration to be a marker of improved prognosis (16,17,54) in the entire cohort both for OS (Figure 3C, Table 2) and for PFS (Figure 3D, Table 2). Both Lin et al. and Kinoshita et al. described high CD8+ TIL infiltration as beneficial in ADC (55,56) and non-ADC (56). However, in our analyses, a high CD8+ TIL infiltration in SCC but not in ADC plus LCC was found to be an independent prognostic factor in stage I–III NSCLC (Tables 3,4).

Regarding FOXP3+ T_T cellular infiltration, our results contribute to the findings of other publications (11,37). Similar to our data, these authors reported less FOXP3+...
TIL infiltration in SCC; however, in ADC (C,D) as well as LCC (E,F) FOXP3+ TILs do not influence OS or PFS. SCC, squamous cell carcinoma; ADC, adenocarcinoma; LCC, large cell carcinoma; OS, overall survival; PFS, progression-free survival; TIL, tumor infiltrating leukocyte.

Table 3

|        | SCC | Mean FOXP3+ TIL infiltration | Log-rank P | Number at risk |
|--------|-----|------------------------------|------------|----------------|
| neg    | 22  | 12  | 10 | 9 | 6 | 4 | 2 | 1 | 0 | 0 | 0 | 0 | 0 |
| pos    | 19  | 11  | 6  | 4  | 2  | 2  | 1  | 0  | 0  | 0  | 0  | 0  | 0  |

Figure 4 OS and PFS for SCC, ADC and LCC depending on FOXP3+ TIL infiltration. (A) OS as well as (B) PFS is influenced by FOXP3+ TIL infiltration in SCC; however, in ADC (C,D) as well as LCC (E,F) FOXP3+ TILs do not influence OS or PFS. SCC, squamous cell carcinoma; ADC, adenocarcinoma; LCC, large cell carcinoma; OS, overall survival; PFS, progression-free survival; TIL, tumor infiltrating leukocyte.

TIL cells in non-squamous NSCLC than in SCC. Here, we identified that specifically ADC but not LCC lack FOXP3+ T-lymphocytic cells (Figure 2A). Moreover, our data indicate that increased evidence of stromal FOXP3+ cells resulted in improved survival in SCC (11) (Table 3 and Figure 4A,B), especially in PD-L1 positive tissue (Figure S2D), but not in ADC (Figure 4C,D) or LCC (Figure 4E,F). At first glance the results for the SCC subcohort are contradictory since many retrospective survival analyses on NSCLC depicted high Treg cell infiltration to be an unfavorable prognostic factor (37-39). The latter findings were reproduced in cell culture: Here, presence of FOXP3+ Treg cells finally enhanced tumor growth and reduced apoptosis in 95D lung cancer cells (57). At the same time, however, in presence of FOXP3+ Treg cells local tumor cell migration was reduced and tumor cell adhesiveness was promoted (57). Hence, the effect of FOXP3+ Treg cells on NSCLC tissue is manifold and this cell-interaction especially in the light of tumoral...
PD-L1 expression needs further investigation. Still, we have to consider, that FOXP3 might transiently be expressed by conventional CD4+ CD25+ T\textsubscript{reg} cells or even cytotoxic CD8+ T\textsubscript{c} cells (24-26,40,41). Therefore, the evaluation of tumoral T\textsubscript{reg} cells becomes even more challenging and might partly explain opposing prognostic evaluations on T\textsubscript{reg} cells in NSCLC.

With focus on the pathogenetic and prognostic impact of FOXP3+ T\textsubscript{reg} cells there is a paradox in solid tumors, outlined by Shang et al. in their meta-analysis (58). While in colorectal cancer (59), in head and neck SCC (33) as well as in esophageal cancer (58) a high proportion of FOXP3+ T\textsubscript{reg} cells resulted in superior OS, it was found reversely for breast cancer, hepatocellular cancer, pancreatic cancer, melanomas, ovarian cancer, renal cell carcinomas and cervical cancer (58). Nevertheless, for follicular lymphoma and Hodgkin's lymphomas, FOXP3+ T cell infiltration was also associated with improved outcome (58). Shang et al. propose a prognostically beneficial role of FOXP3+ T\textsubscript{reg} cells in heavily granulocyte and macrophage infiltrated solid tumors with elevated amounts of cytokines (58). Of interest, in our study cohort TIL infiltration was non significantly higher in SCC compared to ADC but FOXP3+ infiltrates were more prominent in SCC and LCC than in ADC (Figure 2A).

In conclusion, unlike other publications (37-39), the present cohort did not reveal any association for high FOXP3+ T\textsubscript{reg} cell infiltration with dismal outcome in stage I–III tumors and the prognostic benefit of high FOXP3+ infiltrates in PD-L1 positive SCC needs further clarification. Nevertheless, high levels of CD8+ cells in the tumor microenvironment indicated improved OS and PFS in the overall cohort and SCC subcohort, especially if PD-L1 negative, while a high CD4+ TIL infiltration was associated with improved OS in the overall cohort as well as in the ADC and LCC subcohort. Yet, a CD8+ dominant T cell pattern was associated with high grade NSCLC tumors. The opposing results of TIL influence on SCC and ADC emphasize the need for further studies of T cell impact on NSCLC, especially against the background of histology, EMT state, PD-L1 expression and molecular biology.

**Acknowledgments**

We thank Inka Buchroth, Lydia Fälker and Christin Fehmer from the Gerhard Domagk Institute of Pathology, University Hospital Muenster for their excellent work on performing immunohistochemistry. 

**Funding:** None.

**Footnote**

**Conflicts of Interest:** All authors have completed the ICMJE uniform disclosure form (available at [http://dx.doi.org/10.21037/jtd-19-3414a](http://dx.doi.org/10.21037/jtd-19-3414a)). The authors have no conflicts of interest to declare.

**Ethical Statement:** The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was performed in concordance with the ethical approval obtained from the concerned Ethical Committee in Münster (Az 2016-445-f-S and Reg.Nr.: 4XMüller1).

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Cite this article as: Schulze AB, Evers G, Görlich D, Mohr M, Marra A, Hillejan L, Rehkämper J, Schmidt LH, Heitkötter B. Tumor infiltrating T cells influence prognosis in stage I–III non-small cell lung cancer. J Thorac Dis 2020;12(5):1824-1842. doi: 10.21037/jtd-19-3414a
**Figure S1** Positive immunohistochemistry controls on human tonsil tissue. Positive staining reaction of the antibodies used. (A) CD4 monoclonal antibody clone SP35 Roche/Ventana in ×200 (left) and ×400 (right) magnification; (B) CD8 monoclonal antibody clone SP57 Roche/Ventana in ×200 (left) and ×400 (right) magnification; (C) FOXP3 polyclonal antibody clone ab4728 Abcam in ×200 (left) and ×400 (right) magnification.
| Clinicopathological characteristics | CD4 Low (<20%), n=161 | CD4 High (>20%), n=133 | P | CD8 Low (<20%), n=139 | CD8 High (>20%), n=155 | P | FOXP3 Neg (0%), n=171 | FOXP3 Pos (≥1%), n=123 | P |
|-----------------------------------|------------------------|-------------------------|---|-----------------------|-------------------------|---|------------------------|-------------------------|---|
| **Age [years]**                   |                        |                         |   |                       |                         |   |                        |                         |   |
| Mean (± SD)                       | 65.93 (±9.31)          | 64.45 (±7.69)           | 0.143 | 65.86 (±9.35)          | 64.72 (±7.93)           |   | 65.49 (±8.76)          | 64.94 (±8.49)           | 0.593 |
| **Sex**                           |                        |                         |   |                       |                         |   |                        |                         |   |
| Male                              | 129 (80.1)             | 105 (78.9)              | 0.885 | 112 (80.6)            | 122 (78.7)              |   | 133 (77.8)             | 101 (82.1)              | 0.383 |
| Female                            | 32 (19.9)              | 28 (21.1)               |   | 27 (19.4)             | 33 (21.3)               |   | 38 (22.2)              | 22 (17.9)               |   |
| **Smoking status (n=293)**        |                        |                         |   |                       |                         |   |                        |                         |   |
| Negative                          | 35 (21.7)              | 32 (24.2)               | 0.675 | 35 (25.4)             | 32 (20.6)               |   | 42 (24.6)              | 25 (20.5)               | 0.481 |
| Positive                          | 126 (78.3)             | 100 (75.8)              |   | 103 (74.6)            | 123 (79.4)              |   | 129 (75.4)             | 97 (79.5)               |   |
| **Histopathology**                |                        |                         |   |                       |                         |   |                        |                         |   |
| SCC                               | 83 (51.6)              | 57 (42.9)               | 0.319 | 74 (53.2)             | 66 (42.6)               |   | 75 (43.9)              | 65 (52.8)               | 0.132 |
| ADC                               | 58 (36.0)              | 55 (41.4)               |   | 46 (33.1)             | 67 (43.2)               |   | 74 (43.3)              | 39 (31.7)               |   |
| LCC                               | 20 (12.4)              | 21 (15.8)               |   | 19 (13.7)             | 22 (14.2)               |   | 22 (12.9)              | 19 (15.4)               |   |
| **Grade**                         |                        |                         |   |                       |                         |   |                        |                         |   |
| G1                                | 1 (0.6)                | 2 (1.5)                 | 0.243 | 2 (1.4)               | 1 (0.6)                 |   | 2 (1.2)                | 1 (0.8)                 | 0.364 |
| G2                                | 44 (27.3)              | 48 (36.1)               |   | 44 (31.7)             | 48 (31.0)               |   | 57 (33.3)              | 35 (28.5)               |   |
| G3                                | 98 (60.9)              | 65 (48.9)               |   | 75 (54.0)             | 88 (56.8)               |   | 92 (53.8)              | 71 (57.7)               |   |
| G4                                | 18 (11.2)              | 18 (13.5)               |   | 18 (12.9)             | 18 (11.6)               |   | 20 (11.7)              | 16 (13.0)               |   |
| **UICC6 pT**                      |                        |                         |   |                       |                         |   |                        |                         |   |
| pT1                               | 47 (29.2)              | 48 (36.1)               | 0.289 | 42 (30.2)             | 53 (34.2)               |   | 53 (31.0)              | 42 (34.1)               | 0.434 |
| pT2                               | 92 (57.1)              | 68 (51.1)               |   | 78 (56.1)             | 82 (52.9)               |   | 93 (54.4)              | 67 (54.5)               |   |
| pT3                               | 16 (9.9)               | 11 (8.3)                |   | 13 (9.4)              | 14 (9.0)                |   | 18 (10.5)              | 9 (7.3)                 |   |
| pT4                               | 6 (3.7)                | 6 (4.5)                 |   | 6 (4.3)               | 6 (3.9)                 |   | 7 (4.1)                | 5 (4.1)                 |   |
| **UICC6 pN**                      |                        |                         |   |                       |                         |   |                        |                         |   |
| pN0                               | 104 (64.6)             | 74 (55.6)               | 0.039 | 84 (60.4)             | 94 (60.6)               |   | 109 (63.7)             | 69 (56.1)               | 0.507 |
| pN1                               | 37 (23.0)              | 26 (19.5)               |   | 29 (20.9)             | 34 (21.9)               |   | 27 (15.8)              | 36 (29.3)               |   |
| pN2                               | 20 (12.4)              | 33 (24.8)               |   | 26 (18.7)             | 27 (17.4)               |   | 35 (20.5)              | 18 (14.6)               |   |
| **UICC6 pTNM stage**              |                        |                         |   |                       |                         |   |                        |                         |   |
| Stage I                           | 92 (57.1)              | 63 (47.4)               | 0.047 | 75 (54.0)             | 80 (51.6)               |   | 97 (56.7)              | 58 (47.2)               | 0.482 |
| Stage II                          | 40 (24.8)              | 33 (24.8)               |   | 32 (23.0)             | 41 (26.5)               |   | 31 (18.1)              | 42 (34.1)               |   |
| Stage III                         | 29 (18.0)              | 37 (27.8)               |   | 32 (23.0)             | 34 (21.9)               |   | 43 (25.1)              | 23 (18.7)               |   |
| **TIL count (%)**                 |                        |                         |   |                       |                         |   |                        |                         |   |
| Mean (± SD)                       | 24.47 (±12.97)         | 29.39 (±13.67)          | 0.002 | 23.48 (±12.42)        | 29.58 (±13.79)          |   | 25.67 (±13.79)         | 28.12 (±12.97)          | 0.125 |
| **PFS (days)**                    | 0.488                  |                         |   |                       |                         |   |                        |                         |   |
| Median [95% CI]                   | 841 [562–1,120]        | 1,056 [600–1,512]       | 0.690 | 699 [438–960]         | 1,322 [685–1,959]       |   | 927 [611–1,243]        | 964 [531–1,397]         |   |
| **OS (days)**                     | 0.10                   |                         |   |                       |                         |   |                        |                         | 0.699 |
| Median [95% CI]                   | 1,092 [797–1,387]      | 1,657 [926–2,388]       |   | 944 [635–1,253]       | 2,019 [1,392–2,645]     |   | 1,257 [993–1,521]      | 1,592 [869–2,315]       |   |
| Follow up (days)                  | 0.649                  |                         |   |                       |                         |   |                        |                         |   |
| Median [95% CI]                   | 2,656 [2,451–2,861]    | 2,747 [2,529–2,965]     |   | 2,681 [2,592–2,770]   | 2,805 [2,523–3,087]     |   | 2,748 [2,524–2,971]    | 2,596 [2,426–2,766]     |   |

* Equal variances assumed via t-test for age and TIL count. SD, standard deviation; SCC, squamous cell carcinoma; ADC, adenocarcinoma; LCC, large cell carcinoma; score; TIL, tumor infiltrating leukocyte; PFS, progression-free survival; OS, overall survival; CI, confidence intervals.
Table S2 Post-hoc correlations of PD-L1 with CD4⁺, CD8⁺ and FOXP3⁺ TIL infiltration and FOXP3 expression with regard to histology

| Variables | Expression characteristics | PD-L1 tumor stain | P      |
|-----------|----------------------------|-------------------|--------|
| A, n=293  |                            |                   |        |
| CD4⁺ Low  | <20%, n=160                | 54 (33.8)         | 0.841  |
| CD4⁺ High | ≥20%, n=133                | 46 (34.6)         |        |
| CD8⁺ Low  | <20%, n=138                | 58 (42.0)         | 0.001  |
| CD8⁺ High | ≥20%, n=155                | 42 (27.1)         |        |
| FOXP3⁻    | Negative (0%), n=170       | 75 (44.1)         | <0.001 |
| FOXP3⁺    | Positive (≥1%), n=123      | 25 (20.3)         |        |
| SCC, n=140| Negative (0%), n=75        | 35 (46.7)         | 0.006  |
| ADC, n=112| Negative (0%), n=73        | 28 (38.4)         | 0.023  |
| LCC, n=41 | Negative (0%), n=22        | 12 (54.5)         | 0.002  |
| B, n=293  | with regard to FOXP3⁺ TILs |                   |        |
| SCC, n=140| Positive (≥1%), n=65       | 16 (24.6)         |        |
| ADC, n=112| Positive (≥1%), n=39       | 5 (12.8)          |        |
| LCC, n=41 | Positive (≥1%), n=19       | 4 (21.1)          |        |

A. Post-hoc association of PD-L1 stain with CD4⁺, CD8⁺, and FOXP3⁺ TILs; B, association of PD-L1 stain FOXP3⁺ TILs with respect of tumor histology. *, P values gained via Mann-Whitney U test. IRS, immunoreactive score; TIL, tumor infiltrating leukocyte; SCC, squamous cell carcinoma; ADC, adenocarcinoma; LCC, large cell carcinoma.
**Figure S2** OS depending on PD-L1 status. CD8⁺ TILs highly influence OS in low PD-L1 expressing tumors (A), but not in high PD-L1 expressing tumors (B); FOXP3⁺ TILs do not influence survival in PD-L1 low expressing SCC (C) but relevantly influence prognosis in PD-L1 overexpressing SCC (D). SCC, squamous cell carcinoma; OS, overall survival; TILs, tumor infiltrating leukocytes.

**Figure S3** OS and PFS for SCC and ADC plus LCC depending on CD4/CD8 ratio. (A) OS as well as (B) PFS is influenced by a high (CD4-dominant) or low (CD8-dominant) ratio neither in ADC and LCC nor in SCC (C,D). SCC, squamous cell carcinoma; ADC, adenocarcinoma; LCC, large cell carcinoma; OS, overall survival; PFS, progression-free survival.