

Narrative review of the prognostic significance of immune cells in the tumor microenvironment of stage I lung cancer

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Abstract: Current staging for lung cancer is primarily based on TNM staging, which is purely anatomical. This staging method has served an important purpose of stratifying patients into risk categories based on tumor physical characteristics including tumor size, nodal involvement and metastasis. Nonetheless, the TNM staging has its limitations. One such limitation is the fact that this staging method cannot prognostically discriminate within the same tumor stage. This issue may become relevant with the increasing number of stage I patients being detected as a result of lung cancer screening. As such, investigations for additional prognostic markers become important. The tumor immune microenvironment (IME), including infiltrating immune cells, cell surface markers of these infiltrating cells and of the tumor cells, and signaling proteins (specifically cytokines), could provide the opportunity to stratify patients with early-stage lung cancer based on prognosis (e.g., post-operative recurrence risk) and provide insight on therapeutic responses as well as therapeutic targets. Knowledge of the IME in cancers is important as it serves as a basis for research that attempts to study the possibility of employing the immune system to actively destroy cancer cells (i.e., cancer immunotherapy). This article aims to review recent findings as they relate to prognosticators in the IME of stage I lung cancer.

Keywords: Immune; microenvironment; lung cancer; tumor cells

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Introduction

Our discussion of the immune microenvironment (IME) in early lung cancer will include the importance of immune cells in the tumor IME. Not only is the presence of certain immune cells relevant, their exact location within the tumor microenvironment also appears to be significant: i.e., within the tumor itself, or in the surrounding stroma, as are the types of infiltrating immune cells [including lymphocytes—B-cells, T-cells (CD4+, CD8+, regulatory T-cells/T-reg), macrophages and neutrophils], the density of immune cells, and the proportion of pro-tumor and anti-tumor

immune cells (1). This article will elaborate on each of these aspects of tumor infiltrating immune cells. We present the following article in accordance with the NARRATIVE REVIEW reporting checklist (available at <http://dx.doi.org/10.21037/amj-20-118>).

Methods

We performed a PubMed search using the following phrases “lung cancer”, “immune prognostic factors”, “stage I”, which provided 88 search results. We further limited the publication dates to the last 10 years: 2009-01-01–2019-12-

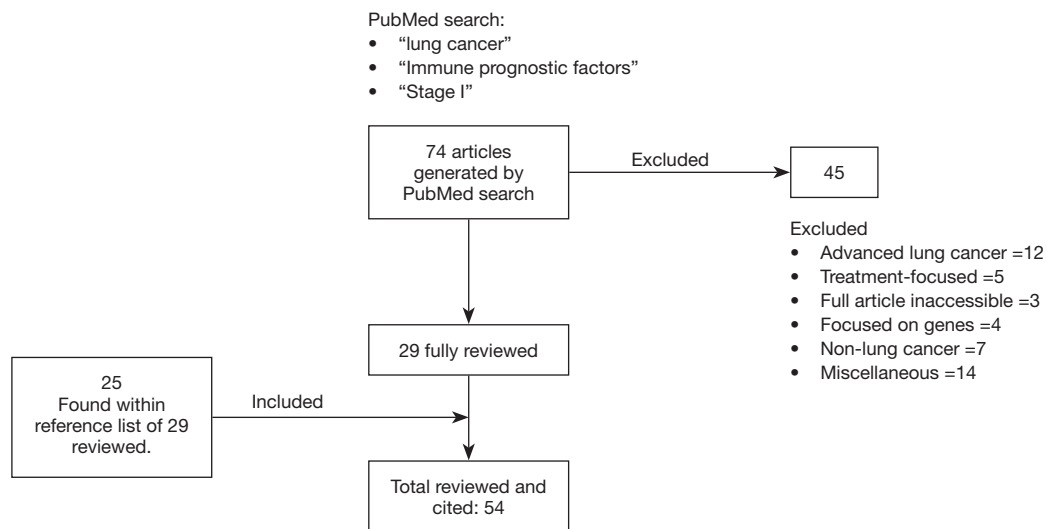


Figure 1 Flow diagram of the literature search process.

31, which filtered the search results down to 74 (Figure 1). We further excluded articles whose primary focus was specifically stated to be advanced stage lung cancer, articles we were unable to get full access to, and articles that were not relevant to the topic at hand, e.g., articles focused on treatment, genetic analysis, non-lung cancer, etc. More relevant articles were found within the reference lists of the previously mentioned relevant articles. In the current review, we will focus on immune cells in the tumor microenvironment. In total we reviewed 54 articles, to obtain a robust summary of the current available knowledge on the IME associated with stage I and early-stage lung cancer.

Discussion

Tumor-infiltrating lymphocytes (TILs), non-specified

Most simple analysis of TILs is an assessment on routine hematoxylin and eosin stains (H&E). Such approach has demonstrated the prognostic value of TILs. An example is a study of 219 patients who underwent lobectomy for stage I non-small cell lung cancer (NSCLC). Tissue samples were stained by routine H&E stain and analyzed by blinded pathologists. The results showed that a moderate-to-severe lymphocyte infiltration of large (≥ 5 cm) stage I tumors (n=39) was associated with a lower disease-recurrence and a higher 5-year disease-free survival (DFS). Specifically, patients with no-to-low TILs had a 35.9% 5-year DFS, compared with 75.6% in patients with moderate-to-high

TILs. Similarly, disease recurrence was noted as 60% and 21% in both groups of patients, respectively (2). Another study analyzed 783 stage I–III NSCLC samples by H&E and showed a significant increase in overall survival (OS) and DFS in patients whose tumors had intense lymphocytic infiltration. The 5-year OS in tumors with intense tumor lymphocyte infiltration and those with non-intense tumor lymphocyte infiltration was 59% and 40%, respectively (P=0.001) while 5-year DFS was 54% and 35% in both groups, respectively (P=0.001). These results were confirmed by similar observation in the validation data set (n=763). This association, although also present in resected adenocarcinoma (ADC) and squamous cell carcinoma (SCC), was greater in non-ADC and non-SCC histological subtypes which included large-cell, adenosquamous, sarcomatoid, basaloid, and unclassifiable NSCLC, in which there was an 88% decrease in the risk of death in patients with intense lymphocytic infiltration, compared with a 38% decrease in the risk of death in the SCC plus ADC groups combined (3). In stage IA NSCLC, similar trends have been shown by Horne *et al.*, in which TILs were shown to infer a significant beneficial association on 5-year DFS in their study population, as well as in a subset group without angiolymphatic invasion. Five-year DFS was 87% in the TIL-positive (TIL+) group, compared with 73% in the TIL-negative (TIL-) group (P=0.011). Furthermore, while there was no statistical difference in locoregional recurrences between the TIL+ and TIL- groups, systemic recurrence was more common in the TIL- group (16.4%)

Table 1 Summary of Tumor-infiltrating lymphocytes in the immune microenvironment of early-stage lung cancer

Author	Stages	n	Conclusion
Kilic <i>et al.</i> (2)	I	219	High infiltration is associated with: Lower disease recurrence Longer 5-year DFS No-to-low TILs in large (>5 cm) stage I tumors was associated with a 35.9% 5-year DFS, and a 60% recurrence, compared with moderate-to-high TILs patients, in whom 5-year DFS was 75.6%, and recurrence rate was 21%
Brambilla <i>et al.</i> (3)	I-III	783	High infiltration is associated with: Longer OS Longer DFS 5-year OS for intense lymphocyte-infiltrated tumors was 59%, compared with 40% in low-infiltrated tumors (P=0.001) 5-year DFS in high-infiltration tumors was 54%, compared with 35% in the low-infiltration group (P=0.001)
Horne <i>et al.</i> (4)	IA	273	High infiltration is associated with: Increased rate of 5-year DFS Decreased systemic recurrence 5-year DFS was 87% in the TIL+ patients, and 73% in the TIL- patients; P=0.011 Systemic recurrence rate was 6.4% in the TIL+ group, and 16.4% in the TIL- group; P=0.0295
Ruffini <i>et al.</i> (5)	I-IIIa	1,290	Increased TIL prevalence trended with increased survival in SCC (P=0.03), but not in other histological subtypes

DFS, disease-free survival; TILs; tumor-infiltrating lymphocytes; OS, overall survival; SCC, squamous cell carcinoma.

than in the TIL+ group (6.4%; P=0.0295). In the subset of patients without angiolymphatic invasion, 5-year DFS was higher in the TIL+ group (93%), than in the TIL- group (79%); P=0.036 (4). Similar results were reported by Ruffini *et al.* in their immunohistochemistry (IHC) study of 1290 resected NSCLC tissues. In this study, a high TIL infiltration correlated with increased cumulative survival in stage I SCC patients (P=0.03), but not in stage I ADC patients (P=0.63), or in the general study population not stratified by histological subtype (5). In summary, TILs have been shown to be associated with prolonged OS and reduced disease recurrence. These studies (*Table 1*) demonstrate the prognostic value of immune response gained from simple H&E; however, they did not investigate specific subtypes of immune cells.

CD8+ T-cells

CD8+ T-cells are referred to as the effector arm of the immune system as they are involved in the cell-mediated

recognition and destruction of abnormal cells, including infected and cancer cells, by recognizing non-self-antigens expressed on major histocompatibility complex class I (MHC I) molecules on aforementioned abnormal cells (6). CD8+ TILs have been shown to infer a good prognosis in lung ADC. Laboratory experiments on murine and human cells have shown that T-cells can kill tumor cells (7). Schalper *et al.* found in their study of stage I-IV NSCLC in 2 independent groups using H&E and multiplex immunofluorescent staining that high CD8+ T-cell infiltration in the tumor microenvironment was independently associated with improved survival (Group 1: P=0.03, 95% CI: 0.280–0.968, HR: 0.533. Group 2: P=0.004, 95% CI: 0.385–0.842, HR: 0.576) (8). Another study on ADC showed that a high CD8+ T-cell infiltration as assessed by IHC was associated with a significantly improved 5-year OS; this survival advantage was even more pronounced when a high CD8+ infiltration was associated with a concurrently high mature dendritic cell infiltration (5-year OS: 45.4% with low CD8+ infiltration, 62.7%

with high CD8+ infiltration, $P=0.002$; 64.4% with high CD8+ and high mature dendritic cells, $P=0.00008$) (9). These findings are supported by other studies, including that by Alifano *et al.*, in which a high intratumoral CD8+ T-cell and mature dendritic cell infiltration, assessed by IHC, were associated with a significant improvement in 5-year OS, including in stage I–II lung cancers. Higher CD8+ infiltration was associated with a 63.3% 5-year OS, compared with 35.7% in low-infiltration tumors ($P=0.0028$); similarly, high mature dendritic cell infiltration was associated with a 5-year OS of 59.2%, compared with 40.2% in low infiltration tumors ($P=0.0015$) (10). Improved DFS and OS were demonstrated by Teng *et al.* in their study, also using IHC on stage I NSCLC tissue samples. DFS showed a positive correlation with increased CD8+ infiltration in both univariate ($P=0.002$, 95% CI: 0.217–0.714, HR: 0.393) and multivariate analyses ($P=0.034$, 95% CI: 0.053–0.892, HR: 0.218). Similar findings were shown for OS in univariate analysis ($P=0.044$, 95% CI: 0.259–0.982, HR: 0.505) although multivariate analysis only showed a trend between high CD8+ TILs and improved OS ($P=0.070$, 95% CI: 0.276–1.052, HR: 0.539) (11). Usó *et al.* assessed the presence of immune cells in 122 stage I–IIIA NSCLC tissue samples, using both IHC and gene expression by means of quantitative polymerase chain reaction (PCR). Both IHC and gene expression analyses showed that a high tumor CD8+ infiltration in NSCLC was associated with improved DFS and OS. Univariate analysis of IHC-derived results showed that a high CD8+ infiltration was associated with a median DFS 56.8 months, compared with a median DFS of 23 months in the low-infiltration group ($P=0.026$). Similarly, improved OS was shown in univariate analysis of IHC data, with high and low CD8+ infiltration being associated with a median OS of 73.9 months and 40.4 months, respectively ($P=0.021$). Univariate analysis of gene expression data also showed a similar positive correlation between high tumoral CD8+ infiltration and DFS (median DFS for high *vs.* low tumoral infiltration: 81.2 *vs.* 19.4 months; $P<0.001$) and OS (median OS for high *vs.* low CD8+ infiltration: 81.2 *vs.* 37.2 months; $P<0.001$). Univariate analysis of gene expression data showed that a high stromal CD8+ infiltration was significantly associated with improved OS, (median OS survival for high *vs.* low stromal CD8+ infiltration respectively: 74.3 months, 46.4 months, $P=0.032$), but not DFS (29.1 *vs.* 25.6 months; $P=0.237$). Multivariate analyses showed that tumoral CD8+ infiltration was an independent prognostic factor for OS and DFS, using both gene expression and IHC data (Gene expression: OS, $P<0.001$,

95% CI: 0.064–0.447, HR: 0.169; DFS, $P<0.001$, 95% CI: 0.120–0.507, HR: 0.247. IHC data analysis: OS, $P=0.018$, 95% CI: 0.175–0.850, HR: 0.386; DFS, $P=0.004$, 95% CI: 0.137–0.680, HR: 0.305) (12). Although several studies have shown CD8+ infiltration to be associated with a better prognosis, there are some studies with contradicting results. Mori *et al.* found in their IHC study of 128 stage I to IIIB NSCLC samples that tumoral CD8+ infiltration was not associated with survival, but that greater CD8+ infiltration was more associated with undifferentiated tumors than with well- or moderately-differentiated tumors ($P<0.05$) (13). Similar findings were also seen in a study by Ilie *et al.*, in which IHC staining was performed on 632 stage I–III NSCLC tissue samples. Progressively higher CD8+ infiltration was seen as tumors became less differentiated. High infiltration in grade 1 to 4 tumors was seen in 43%, 52%, 54% and 65% of patients, respectively (14). These results are also supported by the study by Wakabayashi *et al.*, in which CD8+ T-cells assessed by IHC in the tumor nests of 178 NSCLC tissues samples were found to be negative prognosticators for survival. CD8+ cells were more frequently found in poorly and undifferentiated tumor nests than in well-differentiated tumor nests ($P<0.01$); 5-year OS was 47% in high CD8+ infiltrated tumor nests, compared with 60% in low CD8+ infiltrated nests ($P=0.03$). When the data was stratified by histological subtype, the survival disadvantage associated with high CD8+ tumoral infiltration was maintained in ADC ($P=0.02$), but not in SCC (15). These prognostic findings were not seen for CD8+ cells in the tumor stroma. Increased CD8+ infiltration has been shown to be negatively correlated with decreased expression of tumor microenvironment programmed death ligand-1 (PD-L1), a transmembrane protein found on the surface of various cells of the immune system, including B- and T- lymphocytes, Natural Killer (NK) cells, activated monocytes, macrophages and dendritic cells, whose functions include regulating the immune response by effects that include promoting regulatory T-cell (T-reg) activity (16,17). Rashed *et al.* in their IHC analysis of various cell molecules in NSCLC, showed that tumors with low levels of CD8+ TILs were associated with greater PD-L1 positivity (75%), compared with high CD8+ TILs tumors, which expressed much lower PD-L1 positivity in the microenvironment (17.9%; $P=0.009$) (18). These findings suggest that the lack of CD8+ infiltration in the IME of lung cancer may be associated with decreased anti-tumoral response by the immune system. In summary, there appears to be a general notion that CD8+ T-cell

infiltration is associated with improved prognosis. Variances between non-corroborating studies may be attributed to a number of reasons, including varying sample sizes, different IHC markers and techniques, lack of standardized cut-offs to define high-vs-low infiltration, and lack of studies investigating the functional status of these cells (Table 2).

CD4+ T-cells

CD4+ T-cells have been described to assume one of two opposing functions, whereby one extreme is composed of CD4+ T-cells responsible for promoting CD8+ effector response, while the other end of the spectrum is composed of CD4+ cells that inhibit CD8+ cell response, and essentially

Table 2 Summary of CD8+ T-cell infiltration in the immune microenvironment of early-stage lung cancer

Author	Stages	n	Conclusion
Teng <i>et al.</i> (11)	I	126	High CD8+ infiltration is associated with: improved DFS in univariate and multivariate analyses, and improved OS in univariate but not multivariate analysis DFS in: Univariate analysis (P=0.002, 95% CI: 0.217–0.714, HR: 0.393); 5-year DFS was ~40% in low-infiltration, vs. ~70% in high infiltration multivariate analyses (P=0.034, 95% CI: 0.053–0.892, HR: 0.218) OS in: Univariate analysis (P=0.044, 95% CI: 0.259–0.982, HR: 0.505); 5-year OS was ~60% in low-infiltration, vs. ~ 80% in high-infiltration Multivariate analysis (P=0.070, 95% CI: 0.276–1.052, HR: 0.539)
Rashed <i>et al.</i> (18)	III–IV	50	High CD8+ infiltration is associated with lower expression of PD-L1 on TCs 75% of low CD8+-infiltrated tumors expressed PD-L1, compared with 17.9% of high CD8+-infiltrated tumors; P=0.009
Schalper <i>et al.</i> (8)	I–IV	552	Increased CD8+ infiltration is associated with improved survival in 2 independent groups; P=0.004, P=0.002 Group 1: ~ 40% 5-year survival probability in low-infiltration, vs. ~ 60% in high-infiltration. Group 2: ~ 20% 5-year survival probability in low-infiltration, vs. ~ 50% in high-infiltration.
Mansuet-Lupo <i>et al.</i> (9)	I–IV	316	High CD8+ infiltration is associated with an increased 5-year OS. 5-year OS for: Low CD8+ infiltration: 45.4% High CD8+ infiltration: 62.7%; P=0.002
Alifano <i>et al.</i> (10)	I–IV	303	High CD8+ infiltration is associated with an increased 5-year OS than low CD8+ infiltration. 5-year OS for: Low CD8+ infiltration: 35.7% High CD8+ infiltration: 63.3%; P=0.0028
Usó <i>et al.</i> (12)	I–IIIA	122	Increased CD8+ tumor infiltration is associated with: improved DFS and improved OS Median DFS of 23 months in low-infiltration, vs. 56.8 months in high-infiltration; P=0.026 Median OS of 40.4 months for low-infiltration, vs. 73.9 months in high-infiltration; P=0.021 Increased CD8+ stromal infiltration is associated with: improved OS Median OS of 46.4 months in low-infiltration, vs. 74.3 months in high-infiltration; P=0.032

Table 2 (continued)

Table 2 (continued)

Author	Stages	n	Conclusion
			CD8+ tumoral infiltration is an independent prognostic factor for OS and DFS
Mori <i>et al.</i> (13)	I–IIIB	128	High CD8+ infiltration is more prevalent in undifferentiated/poorly differentiated tumors than in well- and moderately differentiated tumors (P=0.05)
Ilie <i>et al.</i> (14)	I–III	632	High CD8+ infiltration is more prevalent in poorly differentiated, than in well- and moderately differentiated tumors Global chi-square test P=0.04 CD8+ infiltration was much greater in grade 2-4 tumors than in grade 1 tumors (P=0.009)
Wakabayashi <i>et al.</i> (15)	I–III	178	High CD8+ tumoral infiltration is associated with: poorly differentiated than well-differentiated tumors (P<0.01) decreased 5-year OS (5-year OS for high vs. low infiltration were 47% and 60%, respectively; P=0.03)
Mansuet-Lupo <i>et al.</i> (9)	I–IV	316	High infiltration by both CD8+ T-cells and mature dendritic cells is associated with improved survival 5-year OS was: 64.4% with high CD8+ and mature dendritic cell infiltration 60.8% with high CD8+ and low mature dendritic cell, or low CD8+ and high mature dendritic cell 36.6% with low CD8+ and low mature dendritic cell infiltration P=0.00008

PD-L1, program death ligand 1; TC, tumor cells; DFS, disease-free survival; OS, overall survival; CI, confidence interval; HR, hazard ratio.

play a regulatory role (i.e., regulatory T-cells) (19). Regulatory T-cells (T-regs) are a subset of CD4+ T-cells with high expression of CD25 and CD127 (19). It follows that the earlier mentioned CD4+ subset may likely play an important anti-tumor role, and the latter may be involved in tumorigenesis and progression (5). T-regs also contain the nuclear transcription factor, Foxp3, which is also used as a marker of this subset of lymphocytes known to suppress the host immune response (19). Mony *et al.* in their analysis of microarray data from the Gene Expression Omnibus (GEO), showed that the increased presence of non-regulatory CD4+ T-cells was associated with a higher non-recurrence (P=0.0001) in stage IA/IB ADC (20). Corroborating results were shown by Wakabayashi *et al.*, in which the 5-year OS was higher in tumors with higher CD4+ infiltration in the cancer stroma (64%), compared with tumors with lower CD4+ stromal infiltration (43% P=0.04) (15). Usó *et al.* had concurring results but in the tumor nest, in which tumoral CD4+ T-cells were associated with better prognosis, and specifically, a longer OS and DFS in stage I–IIIA NSCLC (CD4+ high *vs.* low tumoral infiltration: median OS: 81.2

vs. 42.9 months, P=0.018; median DFS: 37.8 *vs.* 23 months, P=0.042) (12).

Regulatory T-cells

T-regs have been shown to be involved in suppressing the immune response (21,22); this effect is a favorable one in terms of preventing the development of autoimmune disorders, but may become adverse in other situations, as appears to be the case in lung cancer. In the study by Usó *et al.*, the ratio of Foxp3 a marker of T-regs, to the total CD4+ T-cell was assessed; when combined as a ratio, a high stromal Foxp3-to-tumour-CD4+ expression inferred a significant reduction in OS (46.6 months) and DFS (19.4 months), compared with a low-stromal Foxp3-to-tumour-CD4+ ratio (OS: 81.2 months, P=0.012; DFS: 37.8 months, P=0.013). These results indicate that increasing the regulatory subset of CD4+ T-cells possibly shifts the immune dynamic towards tumor growth and progression (12). This disadvantageous effect of T-regs in the cancer IME has been shown in further studies. Mony *et al.*

analyzed the patterns in immune composition associated with recurrence in NSCLC (specifically ADC), using microarray data derived from the GEO, and showed that a high presence of T-regs was associated with increased recurrence ($P=0.0048$); a low proportion of macrophages/monocytes was associated with increased non-recurrence ($P=0.0047$), and a greater ratio of T-reg-to-total-T-cell increased the probability of recurrence ($P=0.0032$) (20). These results are in harmony with the current knowledge of the functions of T-regs in diminishing the responses of effector T-cells, including their antitumoral activities (21,22). Studies have shown that a low stromal Foxp3 confers a prognostic advantage over a high stromal Foxp3, which is associated with a shorter DFS. Specifically, Suzuki *et al.* showed in their IHC study of 956 stage I lung ADC tissue samples that a higher 5-year DFS was associated with a low stromal Foxp3 presence (85%), compared with the group of patients with a high stromal Foxp3, which was associated with a 5-year DFS of 80% ($P=0.043$) (23,24). Furthermore, a high stromal CD3 (i.e., overall T-cell) infiltration became protective against a high stromal Foxp3, leading to a higher DFS. Five-year DFS in patients with a high stromal Foxp3-to-stromal CD3+ ratio was 77%, compared with patients with a low stromal Foxp3-to-stromal CD3+ ratio, who had a 5-year DFS of 85%; $P=0.004$ (23). The notion that increased T-regs in the tumor microenvironment is associated with tumorigenesis is further supported by another study that revealed that a high stromal Foxp3 infiltration and a high Foxp3/CD3+ ratio, as assessed by IHC analysis, were associated with increased tumor budding, as assessed by H&E staining. Tumor budding refers to the presence of lone tumor cells or small collections of less than 5 tumor cells, at the outer margin of the tumor, in the tumor stroma, indicative of increased tumor invasiveness and negative prognosis. This study showed that high-grade tumor budding correlated with increased stromal Foxp3 ($P<0.001$) and was associated with an increased cumulative recurrence incidence (CRI). High-grade tumor budding was seen in 41% of tumors with high stromal Foxp3 infiltration, compared with 26% of tumors with low stromal Foxp3 infiltration ($P\leq 0.001$). There were similar trends with tumoral Foxp3+ infiltration, but the findings were not significant ($P=0.37$). Tumors with high-grade budding were associated with a higher 5-year CRI of 32%, compared with low-grade budding (12%, $P<0.001$). This finding was reproduced in the validation dataset, in which 5-year CRI was 20% in the high-grade budding tumors, compared with 12% in the low-grade budding

tumors; $P=0.005$ (25). Another study performed by IHC on resected stage I-III NSCLC tissues, showed that increased Foxp3+ tumor infiltration (>3 cells) was associated with worse DFS, compared with patients with low tumor Foxp3+ (<3 cells) infiltration ($P=0.004$). Furthermore, Foxp3 expression was an independent predictor of DFS following multivariate analysis, in which higher levels of Foxp3 T-cells were associated with shorter DFS ($P<0.016$, 95% CI: 1.38–21.07, HR: 5.38) (26). Foxp3 infiltration has also been shown to correlate with increased expression of nuclear estrogen-receptor- α in male lung ADC and subsequently increased risk of recurrence (27). A positive correlation has also been shown between Foxp3 infiltration and intratumoral cyclooxygenase expression (26). This could be explained by another study that showed that tumoral COX-2 induces Foxp3 expression, leading to increased activity of T-regs in lung cancer (28). This may, at least in part, explain the poorer prognosis in terms of DFS seen in the study by Shimizu *et al.* (26), since COX-2 elaborates prostaglandin E2 (PGE2), which increases the presence of T-regs, which in turn suppress the immune response against tumors (Figure 2). In the study by Shimizu *et al.* (26), poorer prognosis was more greatly highlighted in patients without nodal metastasis. Specifically, the DFS in node-negative patients with high Foxp3 infiltration was significantly worse than in node-negative patients with low-to-no Foxp3 infiltration ($P<0.001$); there was no significant difference in DFS between high and low Foxp3 infiltration in the node-positive group ($P=0.688$), possibly hinting that Foxp3 cells may play a more important role in the early-stage tumors (26). The finding that tumor-infiltrating Foxp3 cells are associated with worse prognosis in terms of recurrence has been reproduced by other studies (11,29-31). Yan *et al.* showed that high expression of Foxp3 was associated with shorter DFS (HR =1.336, $P=0.031$), and OS (HR =1.566, $P=0.002$) in stage I-IV NSCLC (31). Teng *et al.* in their study of stage I NSCLC revealed that Foxp3 T-regs were not independent prognosticators of survival, but rather low levels in combination with high CD8+ TILs, were associated with a longer DFS. Low-Foxp3/high-CD8+ TILs was associated with a better DFS than low-Foxp3/low-CD8+ ($P=0.048$) and high-Foxp3/low-CD8+ ($P=0.017$). However, this prognostic advantage of low-Foxp3 was attributed to the associated high levels of CD8+ TILs since there was no significant difference between patients with low-Foxp3/low-CD8+ and high-Foxp3/low-CD8+ ($P=0.555$), and also no difference between patients with high-Foxp3/high-CD8+ and low-Foxp3/high-CD8+ ($P=0.501$) (11).

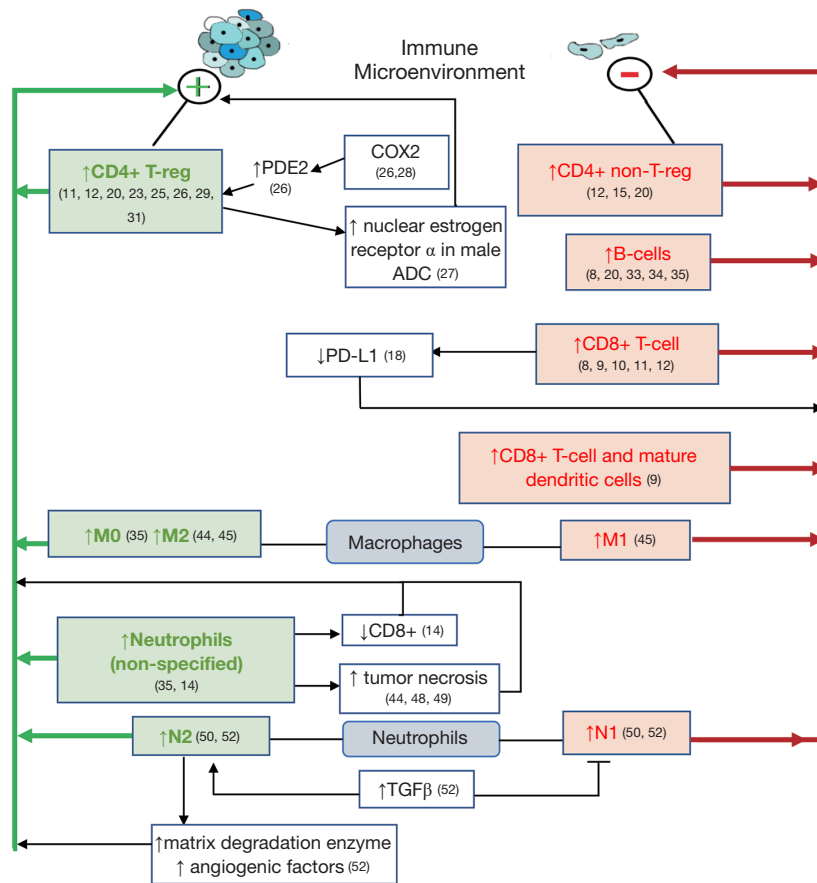


Figure 2 Summary of the prognostic significance of immune cells in the immune microenvironment of lung cancer. ⊕, protumor associations; ⊖, antitumor associations. ↑, increased cell infiltration or increased presence of immune factor; ↓, decreased cell infiltration or decreased presence of immune factor. COX2, cyclooxygenase 2; PDE2, prostaglandin E-2; TGFβ, transforming growth factor beta.

One mechanism by which the immunosuppressive effect of T-regs is maintained in lung cancer has been suggested to be via the transmembrane glycoprotein, glycoprotein A repetitions predominant (GARP), which maintains the regulatory functions of T-regs, and has been shown to be associated with advanced disease when expressed on Foxp3+ T-regs (32). In general, it appears that increased expression of T-regs in lung cancer is associated with worse prognosis, including shorter OS and DFS, and that ratios of these T-regs to other cells (CD3, CD4, and CD8) have prognostic value. On the other hand, increased non-T-reg CD4+ T-cell infiltration may be associated with improved OS and DFS (Table 3).

B-lymphocytes

The presence of B-cells in the tumor IME can be

recognized by their markers which include CD19, CD20 and CD79A, amongst others. B-lymphocytes have been shown to be increased in lung ADCs that show an increased expression of TTC21A, a gene whose upregulation has been shown to be associated with favorable prognosis by Wang *et al.* (33). This data analysis study using data derived from The Cancer Genome Atlas (TCGA) specifically showed that a high expression of TTC21A was associated with longer OS (P=0.002). Increased TTC21A expression was an independent prognosticator of improved survival (P=0.036, 95% CI: 0.623–0.984, HR:0.783). B-cells were more prevalent in tumors with high expression of TTC21A than in tumors with low expression (P=0.035) (33). One of the mechanisms by which tumor infiltrating B-cells are thought to contribute to the favorable prognosis of lung cancers is by secreting tumor-specific antibodies that boost T-cell response (33). The antitumoral effects of B-lymphocytes

Table 3 Summary of CD4+ (T-regs and non-T-regs) infiltration in the immune microenvironment of early-stage lung cancer

Immune Cell	Author	Stages	n	Conclusion
CD4+ non-regulatory	Mony <i>et al.</i> (20)	IA/IB	280	High infiltration is associated with: Longer DFS (P=0.0001)
	Wakabayashi <i>et al.</i> (15)	I-III	178	Higher stromal infiltration is associated with increased 5-year OS (64%), than lower stromal infiltration (5-year OS =43%; P=0.04)
	Usó <i>et al.</i> (12)	I-IIIA	122	High CD4+ infiltration is associated with: Longer DFS Longer OS Tumors with high vs. low CD4+ infiltration respectively had: Median OS: 81.2 vs. 42.9 months, P=0.018 Median DFS: 37.8 vs 23 months, P=0.042
T-regs/Foxp3	Mony <i>et al.</i> (20)	I	280	High prevalence of T-regs is associated with increased recurrence (P=0.0048) Increased T-reg-to-total-T-cell ratio is associated with increased probability of recurrence (P=0.0032)
	Usó <i>et al.</i> (12)	I-IIIA	122	High stromal Foxp3-to-tumour-CD4+ expression is associated with a reduced OS and DFS OS for high vs. low ratio: 46.6 vs. 81.2 months; P=0.012 DFS for high vs. low ratio: 19.4 vs. 37.8 months; P=0.013
	Suzuki <i>et al.</i> (23)	I	956	High stromal Foxp3 is associated with an increased risk of recurrence Low stromal Foxp3 is associated with a higher 5-year RFP (85%), compared with high stromal Foxp3, which was associated a lower 5-year RFP (80%). P=0.043
	Shimizu <i>et al.</i> (26)	I-III	100	High stromal Foxp3 tumor infiltration is associated with a shorter DFS (P=0.004), and shown to be an independent prognostic factor for DFS (P<0.016, 95% CI: 1.38 – 21.07, HR: 5.38)
	Peterson <i>et al.</i> (29)	I	64	A high Foxp3-to-CD3 ratio is associated with shorter survival and was shown to be an independent prognosticator for survival following multivariate analysis. P=0.007, 95% CI: 1.8–38.4, HR: 8.2 Median survival for low Foxp3-to-CD3 ratio tumors was >72 months, compared with 53 months for high Foxp3-to-CD3 ratio tumors (P=0.0148)
	Teng <i>et al.</i> (11)	I	126	Low Foxp3 infiltration in combination with high CD8+ infiltration is associated with longer DFS Low Foxp3/high-CD8+ TILs was associated with a better DFS than low-Foxp3/low-CD8+ (P=0.048) and high-Foxp3/low-CD8+ (P=0.017).
	Kadota <i>et al.</i> (25)	I	1038	High stromal Foxp3+ infiltration correlates with high-grade tumor budding, which correlates with increased risk of recurrence High-grade tumor budding was seen in in 41% of tumors with high stromal Foxp3 infiltration, compared with 26% of tumors with low stromal Foxp3 infiltration; P<0.001 Tumors with high-grade budding were associated with a higher 5-year cumulative recurrence incidence (CRI) of 32%, compared with low-grade budding (12%, P<0.001).
	Yan <i>et al.</i> (31)	I-IV	107	Higher Foxp3 expression in TILs is associated with shorter DFS (HR=1.336, P=0.031) and OS (HR=1.566, P=0.002)

DFS, disease-free survival; OS, overall survival; T-reg, regulatory T-cell; RFP, recurrence-free probability; CI, confidence interval; HR, hazard ratio; TIL, tumor-infiltrating lymphocyte.

have also been reproduced by other studies, including that by Mony *et al.* (20). This study identified 2 subsets of patients with the highest risk of recurrence. One of these subsets included those patients with a high T-reg and macrophage/monocyte presence, and the second subset of patients with highest recurrence were those whose tumors had a low plasma cell (antibody-secreting B-cells) and non-T-reg CD4+ T-cell levels ($P=0.0041$). Plasma cells were further shown to be inversely correlated with recurrence ($P=0.007$) (20). Pelletier *et al.* in their prospective study of 113 stage I–IV NSCLC samples using histological stains (H&E, period acid Schiff, and mucicarmine), flow cytometry and IHC, found a lack of significant relationship between survival and the various prognostic markers they assessed, including T-lymphocyte infiltration. However, the study discovered a significant independent prognostic relationship between tumor-margin B-cells (stained using CD20 marker), and survival ($P=0.04$). Multivariate analysis continued to show that the presence of peritumoral B-cell infiltration was associated with improved survival ($P=0.04$, 95% CI: 0.06–0.42, HR: 0.16). The prognostic advantage associated with tumoral B-cell infiltration was specifically evident in non-SCC patients, who had better survival than patients with the same non-SCC histology without B-cell infiltration ($P<0.001$, 95% CI at 8 years: 0.04–0.40). The presence of B-cell infiltration in SCC was not found to be a significant prognostic factor (34). The results of these studies support the notion that humoral immunity plays an important antitumoral role in lung cancer. The positive prognostic relationship between tumor infiltration by B-cells in NSCLC and survival has also been portrayed by other studies (8,35). Schalper *et al.* performed a study on stage I-IV NSCLC, using 2 independent groups from 2 separate institutions. B-cells were stained for by immunofluorescent techniques, using their CD20 marker. This study showed that increased B-cell infiltration was associated with improved survival; this finding was significant in group 1 ($P=0.004$, 95% CI: 0.323–0.817, HR: 0.523), but not group 2 ($P=0.293$, 95% CI: 0.643–1.236, HR: 0.887). In group 1, 5-year survival probability was approximately 40% and 60% in the low- and high-infiltration groups respectively. Multivariate analysis continued to show B-cell infiltration to be an independent favorable prognostic factor in the earlier group ($P=0.003$, 95% CI: 0.222–0.754, HR: 0.418) (8). All of these findings suggest that humoral immunity plays an important antitumoral role in the IME of lung cancer (Table 4).

Macrophages

Macrophages play different roles in the immune system depending on the functional category assumed by the macrophages. Based on this premise, macrophages may be divided into 1 of 3 types: M0 macrophages (so called “resting macrophages”), M1 macrophages, also known as classically activated macrophages, which assume pro-inflammatory and anti-tumorigenic roles, and M2 macrophages, known as alternatively activated macrophages, with anti-inflammatory and pro-tumorigenic properties (36). Based on this understanding, it may be inferred that certain subtypes of macrophages may be associated with better and others poorer prognosis in lung cancer; however, various studies have produced differing results that do not necessarily align with the basic understanding of the different functions of macrophages. These variances between studies might be explained by the fact that macrophages do not tend to wholly assume an M1 or M2 phenotype *in vivo*, while losing the phenotype of the other subset, but rather adapt more characteristics that allow them to be classified as either M1 or M2, while still retaining some characteristics of the other subset, albeit more poorly expressed (37). Markers commonly used to identify macrophages include cell surface markers, cytokines and transcriptional factors, examples of which, regardless of subset, include but are not limited to: CD14, CD16, CD68, CD86 and HLA-II (37). M1 may be identified by: CD80, IL-1R, TLR2, TLR4, IL-12p70, and others. M2 macrophages may be identified by: CD163, CD200R, MMR/CD206, TLR1, TLR8, IL-1R II and others (37–40). Liu *et al.* in their data analysis-based study of ADC and SCC data derived from TCGA revealed that M0 macrophages were associated with decreased OS ($P=0.049$, 95% CI: 1.01–1.81, HR: 1.35) in early-stage lung ADC, while neither M1 nor M2 was associated with prognosis (35). A higher density of CD68 [a marker applied to all macrophages (41)] has also been found to be associated with poor prognosis in other human cancers (42,43). In lung cancer, Carus *et al.* discovered in their study of NSCLC ($n=335$) that metastasis was significantly higher in patients with increased tumor nest CD163+ macrophage and stromal CD163+ macrophage density ($P<0.05$), however, no survival associations were found (44). CD163+ is a marker of M2 macrophages (41). This finding supports the notion that M2 macrophages, and not M1 macrophages are protumor (45). It appears that increased tumoral infiltration by macrophages, regardless of their subset, may be associated

Table 4 Summary of B-lymphocyte infiltration in the immune microenvironment of early-stage lung cancer

Author	Stages	n	Conclusion
Wang <i>et al.</i> (33)	I–IV	535	Higher B-cell infiltration was associated with higher TTC21A expression, which was shown to be an independent prognosticator of survival B-cells were more prevalent in tumors with high expression of TTC21A than in tumors with low expression (P=0.035) Low levels of TTC21A were associated with shorter survival on univariate analysis (P<0.046, 95% CI: 0.633–0.996), HR: 0.794) Low levels of TTC21A were shown to be independently associated with shorter survival in multivariate analysis (P=0.036, 95% CI: 0.623–0.984, HR:0.783).
Mony <i>et al.</i> (20)	I	280	Tumors with a low plasma cell and non-T-reg CD4+ infiltration combined, are associated with an increased recurrence (P=0.0041) High plasma cell infiltration is associated with decreased recurrence (P=0.007)
Pelletier <i>et al.</i> (34)	I–IV	113	High B-cell infiltration was shown to be associated with improved survival in multivariate analysis. P=0.04, 95% CI: 0.06–0.42, HR: 0.16
Schalper <i>et al.</i> (8)	I–IV	552	Increased B-cell infiltration is associated with improved survival (P=0.004) Univariate analysis: P=0.004, 95% CI: 0.323–0.817, HR: 0.523 5-year survival probability was approximately 40% and 60% in the low- and high-infiltration groups respectively Multivariate analysis: P=0.003, 95% CI: 0.222–0.754, HR: 0.418
Liu <i>et al.</i> (35)	‘Early clinical stage’ (not specified)	980	Increased memory B-cell infiltration (but not plasma cells: P=0.942) was associated with improved survival in lung ADC; P=0.047, 95% CI: 0.55 - 0.97, HR: 0.74 This survival advantage trended in the SCC group, but was not significant. Plasma cell, P=0.099; memory B-cell, P=0.372

CI, confidence interval; HR, hazard ratio; non-T-reg, non-regulatory T-cell; ADC, adenocarcinoma; SCC, squamous cell carcinoma.

with worse prognosis; however, further research into the functions of the different phenotypes of macrophages in lung cancer, may be warranted (*Table 5*).

Neutrophils

Neutrophils are effector cells of the innate immune system, with important roles in acute and chronic inflammation, as well as adaptive immunity. They are identified by high expression of markers such as CD11, CD13, CD15, CD63, CD66, myeloperoxidase, and others (46,47). Liu *et al.* showed in their study using data from TCGA that increased neutrophilic infiltration in SCC was associated with decreased survival (P=0.009, 95% CI: 1.01–1.75, HR: 1.33); similar findings were not found in lung ADC (35). Carus *et al.* showed that intra-tumoral neutrophil (CD66+) density showed a positive correlation with T-stage (P=0.025), degree of necrosis (P<0.0001), and a greater association

with SCC than ADC (P<0.05); however, no associations with OS or DFS were found (44). Although this study did not find any direct correlation between neutrophils and prognosis in terms of recurrence and survival, other studies have shown neutrophil-mediated tumor necrosis to be associated with poorer prognosis. Park *et al.* showed in their retrospective analysis of stage IA NSCLC, using H&E-stained tumor samples, that tumor necrosis was associated with a shorter 5-year OS and shorter 5-year DFS. In necrosis-negative samples, 5-year OS and DFS were 94.8% and 92.1% respectively, while OS and DFS findings for necrosis-positive patients were 86.2% (P=0.04) and 78.9% (P=0.016), respectively (48). Swinson *et al.* in their study on stage I–III NSCLC showed an increased hazard ratio (HR) when extensive tumor necrosis was present (HR: 1.76), compared with no-to-limited tumor necrosis (HR: 1, 95% CI: 1.24–2.50, P=0.0016) (49). This study also analyzed H&E-stained tumor samples. Samples were stratified

Table 5 Summary of macrophage infiltration in the immune microenvironment of early-stage lung cancer

Author	Stages	n	Conclusion
Liu <i>et al.</i> (35)	'Early clinical stage' (not specified)	980	High infiltration of M0 macrophages were associated with poorer OS (P=0.049, 95% CI: 1.01–1.81, HR: 1.35) Neither M1 nor M2 macrophages were found to be prognostic for survival (P=0.139 and 0.877, respectively)
Suzuki <i>et al.</i> (23)	I	956	Low density of macrophages (non-specified) was inversely related to IL-12R/β2 expression (P=0.006), a positive prognostic factor that is positively correlated with longer DFS (5-year DFS in tumors with low vs. high expression of IL-12R/β2 was 80% and 90%, respective; P=0.026)
Carus <i>et al.</i> (44)	I–IIIA	335	Increased M2 macrophages in the tumor nest and stroma (denoted by the CD163 marker) was associated with metastasis (P<0.05), but showed no other prognostic associations

OS, overall survival; CI, confidence interval; HR, hazard ratio; IL, interleukin; DFS, disease-free survival.

according to the degree of necrosis, where samples with a score of 0 had no necrosis and more extensive necrosis was associated with higher scores. The hazard ratio (HR) for samples scored 0–2 was 1, while samples scoring 3–5 had a HR of 1.76 (95% CI: 1.24–2.50; P=0.0016) (49). The findings of these aforementioned studies suggest that neutrophilic infiltration may be associated with fast-growing tumors that outgrow their blood supply, with resulting tumor necrosis. Nevertheless, studies have shown that neutrophils are polarized into one of 2 phenotypes, similar to macrophages. Neutrophils may either assume the N1 or N2 phenotype, where the former has been shown to be involved in mediating antitumor response through mechanisms such as increasing intratumoral CD8+ T-cells, and the latter (N2) has been shown to be protumor and involved in depleting intratumoral CD8+ T-cells (50). TGF-β has been shown to be implicated in neutrophil polarization. This immunosuppressive cytokine is not only involved in suppressing CD8+ T-cell action (51), but also in steering the polarization of neutrophils towards the N2 phenotype, while blocking the elaboration of N1 neutrophils. The N2 neutrophilic phenotype has been suggested to mediate its protumor effects by causing the release of matrix-degrading enzymes and angiogenic factors, which will subsequently promote metastatic features and increased tumoral growth (52). Piccard *et al.* have provided a good summary of the mechanisms of the antitumor and protumor activities of the N1 and N2 neutrophilic subsets, respectively (53). This knowledge of the proposed phenotypes of neutrophils indicates that antitumoral response could be achieved by either stimulating the N1 phenotype or blocking the N2 phenotype. Fridlender *et al.* have suggested that treatment-naïve tumors are likely to

be infiltrated by N2 neutrophils, meaning that depletion of these neutrophils will have an antitumor response, while tumors exposed to some form of immunologic therapy acquire N1-polarised neutrophils, indicating that neutrophil depletion in these tumors will likely lead to a protumor response (50). Ilie *et al.* found in their study of stage I–III NSCLC specimen using IHC, that a high intratumoral CD66b+ cells (i.e., neutrophils) correlated with low levels of CD8+ cells (P=0.016) (14). This study also revealed that high intratumoral infiltration by CD66+ cells was associated with increased rate of recurrence. Median recurrence rate for high CD66+ tumoral infiltration and low CD66+ infiltration was 36 and 51 months, respectively (P=0.002). Median OS for high vs. low CD66+ tumoral infiltration was 54 months and 57 months, respectively (P=0.088) (14). Furthermore, a high intratumoral neutrophil-to-lymphocyte ratio (NLR) was associated with decreased OS and increased rate of recurrence (median survival was 43 months for NLR <1, and 34 months for NLR >1; P<0.0001) (14). This may possibly indicate that a high tumoral neutrophil infiltration may blunt any potential CD8+ effector antitumoral effects. It has been suggested that tumors secrete chemotactic chemicals that increase the presence of neutrophils in tumors; these neutrophils appear to promote tumor growth rather than regression. This leaves the question as to whether tumors recruit N2 neutrophils, or non-N2 neutrophils are recruited and subsequently converted into neutrophils with protumoral N2 capabilities. Some studies have shown that tumor-associated neutrophils (TANs) in early lung cancer do not demonstrate suppression of effector T-cells, and were even shown to stimulate T-cells *in vitro* (54). This suggests that the second theory might be applicable in early-stage lung cancer. The knowledge of the

Table 6 Summary of neutrophil infiltration in the immune microenvironment of early-stage lung cancer

Author	Stages	n	Conclusion
Liu <i>et al.</i> (35)	'Early clinical stage' (not specified)	980	Increased neutrophilic infiltration in SCC is associated with decreased survival (P=0.009, 95% CI: 1.01–1.75, HR: 1.33); similar findings were not found in lung ADC (P=0.343)
Carus <i>et al.</i> (44)	I–IIIA	335	Increased tumoral, peritumoral and stromal neutrophilic infiltration are significantly associated with a higher degree of tumor necrosis (P<0.0001)
Park <i>et al.</i> (48)	IA	201	Tumor necrosis was associated with a shorter DFS in stage IA NSCLC patients In necrosis-negative samples, 5-year OS and DFS was 94.8% and 92.1% respectively, while values for necrosis-positive patients were 86.2% (P=0.04) and 78.9% (P=0.016), respectively
Swinson <i>et al.</i> (49)	I–III	178	A greater degree of necrosis is associated with an increased hazard ratio Tumors with a low degree of necrosis had a HR of 1, while tumors with more extensive necrosis had a HR of 1.76 95% CI: 1.24–2.50; P=0.0016
Ilie <i>et al.</i> (14)	I–III	632	High neutrophil (CD66+) infiltration, correlated with low CD8+ infiltration (P=0.016) High intratumoral neutrophil (CD66+) infiltration was associated with shorter OS and increased recurrence rate A high neutrophil-to-lymphocyte ratio was associated with worse survival Median recurrence rate for high CD66+ infiltration and low CD66+ infiltration was 36 and 51 months, respectively; P=0.002 Median OS for high vs. low CD66+ tumoral infiltration was 54 months and 57 months, respectively; P=0.088 Median survival was 43 months for a neutrophil-to-lymphocyte ratio (NLR) <1, and 34 months for NLR >1; P<0.0001

SCC, squamous cell carcinoma; CI, confidence interval; HR, hazard ratio; ADC, adenocarcinoma; DFS, disease-free survival; OS, overall survival.

functions of TANs in the IME provides possible therapeutic targets that require further research (*Table 6*).

Conclusions

The general consensus from our review is that T-cell (non-specified) infiltration in NSCLC is associated with improved outcome and may play an important role in preventing distant metastasis. A high CD8+ T-cell infiltration of the tumor and its stroma appears to support survival and stave off disease recurrence. High infiltration by non-regulatory CD4+ T-cells appears to have similar antitumoral effects as CD8+ T-cells. Conversely, CD4+ T-regs seem to have an opposite effect, in that they have been shown to promote tumorigenesis and tumor progression. B-lymphocytes have been suggested to play an antitumoral role in lung cancer, possibly by elaborating antitumor antibodies that aid the immune system in tumor destruction. Macrophages appear to be associated with poor survival and increased recurrence;

however, further studies may be required to further assess if the specific subsets of macrophages (M0, M1, and M2) have different prognostic effects in lung cancer. Neutrophils, similar to macrophages have been shown to be capable of assuming one of 2 polarized forms (N1, N2), with the N1 form being associated with an antitumor response, and the N2 form being associated with protumoral properties. Several studies have shown increased neutrophilic infiltration in the tumor IME to be associated with poorer prognosis, while another study showed that neutrophils in early lung cancer possess antitumoral properties, suggesting that polarization from antitumoral to protumoral neutrophils may occur with progression of lung cancer; more research may be warranted in this respect. The knowledge of the roles of immune cells in the tumor IME provides opportunities for potential therapeutic targets as well as clinical prognostication. Further research is still needed to clarify the roles of some cells and/or their subsets in the IME and their clinical usefulness as prognostic indicators (*Figure 2, Table 7*).

Table 7 Summary of pro- and anti-tumor immune cells in the immune microenvironment of early-stage lung cancer

Effect in the IME	Cell	Author	
Antitumor	CD8+ T-cell	Schalper <i>et al.</i> (8)	
		Mansuet-Lupo <i>et al.</i> (9)	
		Alifano <i>et al.</i> (10)	
		Teng <i>et al.</i> (11)	
		Usó <i>et al.</i> (12)	
	Rashed <i>et al.</i> (18)		
	CD4+ non-T-regulatory T-cell	Usó <i>et al.</i> (12)	
		Wakabayashi <i>et al.</i> (15)	
		Mony <i>et al.</i> (20)	
		B-lymphocytes	Schalper <i>et al.</i> (8)
Mony <i>et al.</i> (20)			
Pelletier <i>et al.</i> (34)			
Liu <i>et al.</i> (35)			
Wang <i>et al.</i> (33)			
CD8+ T-cell	Mori <i>et al.</i> (13)		
	Ilie <i>et al.</i> (14)		
	Wakabayashi <i>et al.</i> (15)		
	Protumor	T-regulatory/Foxp3 T-cells	Teng <i>et al.</i> (11)
			Usó <i>et al.</i> (12)
Mony <i>et al.</i> (20)			
Suzuki <i>et al.</i> (23)			
Kadota <i>et al.</i> (25)			
Shimizu <i>et al.</i> (26)			
Peterson <i>et al.</i> (29)			
Yan <i>et al.</i> (31)			
Macrophages		Suzuki <i>et al.</i> (23)	
		Liu <i>et al.</i> (35)	
	Carus <i>et al.</i> (44)		
Neutrophil	Ilie <i>et al.</i> (14)		
	Liu <i>et al.</i> (35)		
	Carus <i>et al.</i> (44)		
	Park <i>et al.</i> (48)		
	Swinson <i>et al.</i> (49)		

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