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Original Article

Synergistic B and T lymphocyte interaction: prognostic implications in non-small cell lung cancer

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Abstract: While T-cell-mediated immune responses in solid tumors have been well-established and have driven major therapeutic advances, our understanding of B-cell biology in cancer is comparatively less developed. A total of 60 lung cancer patients were included, of which 53% were diagnosed at an early stage while 47% were diagnosed at an advanced stage. Flow cytometry was used to analyze the proportion of T and B cells in all blood samples, and the levels of human serum cytokines were also assessed. Compared to the control group, cancer patients showed lower frequencies of IgD+CD27+ marginal B cells and CD32+ B cells, and higher frequencies of T cells with lower CD8+ T cells and higher central memory and naïve CD4+ T cells. Additionally, advanced-stage cancer patients exhibited higher levels of cytokines, a higher proportion of effector memory CD8+ T cells, and a lower frequency of CD27+CD28+CD4+/CD8+ T cells. Linear regression analysis revealed significant correlations between cancer stage and the frequency of B and T cell subsets, leukocyte count, and cytokine levels. Survival analysis demonstrated that patients with higher frequency of class-switched B cells had a worse prognosis, while patients with higher frequency of CD8+ effector T cells and lower frequency of CD4+57+ T cells appeared to have a better survival rate. These findings provide valuable insight into the immunological changes that occur during lung cancer progression and have the potential to inform the development of new immunotherapeutic strategies.

Keywords: Lung cancer, T-cell, B-cell, tumor immunology, cancer immunotherapy

Introduction

Metastatic lung cancer is a significant global health issue, responsible for a substantial number of cancer diagnoses and deaths worldwide. Over the years, the treatment of advanced lung cancer has undergone significant changes, shifting from chemotherapy to targeted molecular agents, and most recently, to immunotherapy strategies [1]. The most promising approach involves using antibodies that block inhibitory receptors on tumor-infiltrating T cells, thereby boosting T cell activity against cancer cells. Specifically, the development of antibodies targeting the programmed death receptor 1 (PD-1), programmed death-ligand 1 (PD-L1), and the cytotoxic T-lymphocyte-associated protein 4 receptor (CTLA-4) has revolutionized the therapeutic approach for metastatic non-small cell lung cancer (mNSCLC) patients. These antibodies have been used in first or second-line settings and have led to unprecedented prolonged survival rates for a portion of these patients [2].

Immune responses within the tumor microenvironment (TME) significantly impact tumor behavior, progression, and aggressiveness [3]. In the TME of lung tumors, T and B cells constitute about two-thirds of immune cells [4]. T-cell-mediated immune responses in solid tumors have been well-established and have driven major therapeutic advances, especially with immune checkpoint inhibitors [5-7]. However, our understanding of B-cell biology in cancer is less developed. Tumor-infiltrating B cells have been observed in all stages of lung cancer development, and their presence varies based on stage and histological subtype [8-10]. However, these investigations predominantly con-

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centrated on the analysis of B cells within the tumor microenvironment. In contrast, the study takes a distinctive approach by centering on the analysis of B cells derived from peripheral blood in lung cancer patients which is necessary. This unique focus allows us to explore the systemic immune response and identify potential circulating biomarkers that could serve as indicators of disease status and prognosis.

As B cells play a role in both humoral and cellular immunity, B-cell parameters may be vital in determining responsiveness to and toxicity of checkpoint blockade. Therefore, manipulating B-cell biology may offer significant immunetherapeutic opportunities [11]. Understanding B-cell biology in non-small cell lung cancer (NSCLC) is crucial to inform potential novel multi-faceted approaches that could reset the immune contexture of the cancer microenvironment favorably [12].

B cells play a crucial anti-tumor role in non-small cell lung cancer [13, 14]. In the peritumoral region, B cells can aggregate to form clusters or tumor-associated tertiary lymphoid structures (TLS) [15]. Plasma cells are also present in tumor infiltrates, although few in number, and they can produce a significant amount of cytokines and antibodies. These antibodies can promote antitumor immunity by facilitating antibody-dependent cellular immunity, opsonization, complement activation, and enhancing antigen presentation by dendritic cells [16]. Furthermore, B cells can present antigens to CD4+ or CD8+ T cells and influence the antigen-specific immune response within the tumor environment. Analyzing the representation of B cell subgroups and molecular subtypes of lung cancer can further optimize and improve the assessment of the prognostic impact of B cells in lung cancer. There is strong evidence that tumor-infiltrating B cells are overwhelmingly favorable prognostic factors in non-small cell lung cancer (NSCLC) patients [11, 13]. However, studies analyzing peripheral B cells in lung cancer patients are limited.

Tumor-infiltrating lymphocytes (TILs) serve as pivotal biomarkers in tumor-immune interactions, with higher concentrations correlating with improved cancer immunotherapy outcomes [17, 18]. However, challenges in TIL quantification, such as limited immunohistochemistry (IHC) methods and inter-observer variability, underscore the need for alternative, accessible predictive markers [19]. Peripheral blood (PB) biomarkers offer promise due to their accessibility and cost-effectiveness, with the neutrophil-to-lymphocyte ratio (NLR) being a well-studied example [20-22]. Yet, the role of peripheral lymphocyte count in cancer immunotherapy remains unclear. Recent research suggests that peripheral T lymphocyte characteristics influence the tumor-immune microenvironment [23], and an increase in PD-1+ CD8 T cell proliferation in blood during treatment may indicate a positive clinical outcome [24]. Identifying prognostic biomarkers to guide patient management is crucial. Thus, our study aims to employ flow cytometry to analyze peripheral T and B cell subpopulations, investigating their prognostic relevance in NSCLC patients.

Methods

Participants

Between 2018 and 2019, we screened patients who were diagnosed with pulmonary nodules and planned to undergo tissue diagnosis. Patients with a pathological diagnosis of lung cancer were enrolled, while the control group was recruited from individuals with normal health. Blood samples were collected before any diagnostic or medical treatment and during a period when participants had no known infections or inflammatory diseases other than lung cancer. The study excluded participants based on recent symptomatic infections, immunizations within the past month, pregnancy, inflammatory diseases, or medication use that could potentially affect the immune system, such as antihistamines or corticosteroids. All participants provided written and informed consent before taking part in the study, which was approved by the local ethics committee (201709028RINA).

The primary objective is to analyze the distribution of peripheral B and T cell subpopulations and investigate the association between B and T lymphocytes. The secondary outcome focuses on determining the prognostic significance of these immune cell subsets in patients with NSCLC.

Sample preparation and flow cytometry

During the study, a 20 mL EDTA-anticoagulated blood sample was collected from each partici-

pant for antibody staining, and another 2 mL sample was sent to the local hospital laboratory for a standard leukocyte cell count. The blood samples were collected before any diagnostic or medical procedure, and during a period when the participants had no identified infection or inflammation diseases other than lung cancer [25]. To identify B cell populations, a panel of 8 markers in fluorochrome combinations was used, which included CD19, CD21, CD24, CD27, CD38, CD45, IgM, and IgD. To identify T cell populations, a panel of 10 markers in fluorochrome combinations was used, which included CD3, CD4, CD8, CD27, CD28, CD45, CD45RA, CD57, CD197 (CCR7), and CD279 (PD-1). All antibodies were obtained from Beckman Coulter (Marseille, France), and analyzed using a cytoflex flow cytometer (Beckman Coulter).

The percentage of CD19+ B cells was determined within the lymphocyte gate using flow cytometry. Further analysis of B cells included assessing their antigen experience, activation, and class-switching status. The frequencies of CD27+ (antigen-experienced) B cells and their CD24hi CD27+ subpopulation were determined, as well as the frequencies of other B cell subsets, including CD27- IgM+ IgDhi (naive), CD27+ IgM- IgD- (switched memory), CD27+ IgM+ IgDlow (unswitched memory), CD27- IgM- IgD- (atypical memory), and IgMhi IgDlow (marginal zone-like) B cells. All samples were analyzed on a 10-color Navios Flow Cytometer (Beckman Coulter) after daily calibration with Flow-Set Pro Beads (Beckman Coulter). The flow cytometry analysis of B and T cells is shown in **Figure 1**.

Multiplex ELISA analysis of cytokines

The levels of human serum cytokines were assessed using the LEGENDplex Custom 12-plex cytokine panel kit (BioLegend, San Diego, CA), which measures IL-1b, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17, TNFα, TGFβ, and TSLP. Serum samples were diluted in assay buffer and cytokine levels were measured on a BD FACS Canto II flow cytometer (BD Biosciences, San Jose, CA) following the manufacturer's protocol. The data obtained were analyzed using LEGENDplex analysis software. The basic characteristics of cytokines were listed in Supplementary Table 1.

Data analysis and statistics

The flow cytometry data analysis was performed using Kaluza version 1.2 (Beckman Coulter) with a standardized gating strategy that was performed by a single operator. Clinical parameters of the patients were recorded, including gender, age, pathological diagnosis, lung cancer stage (AJCC 8), and survival time.

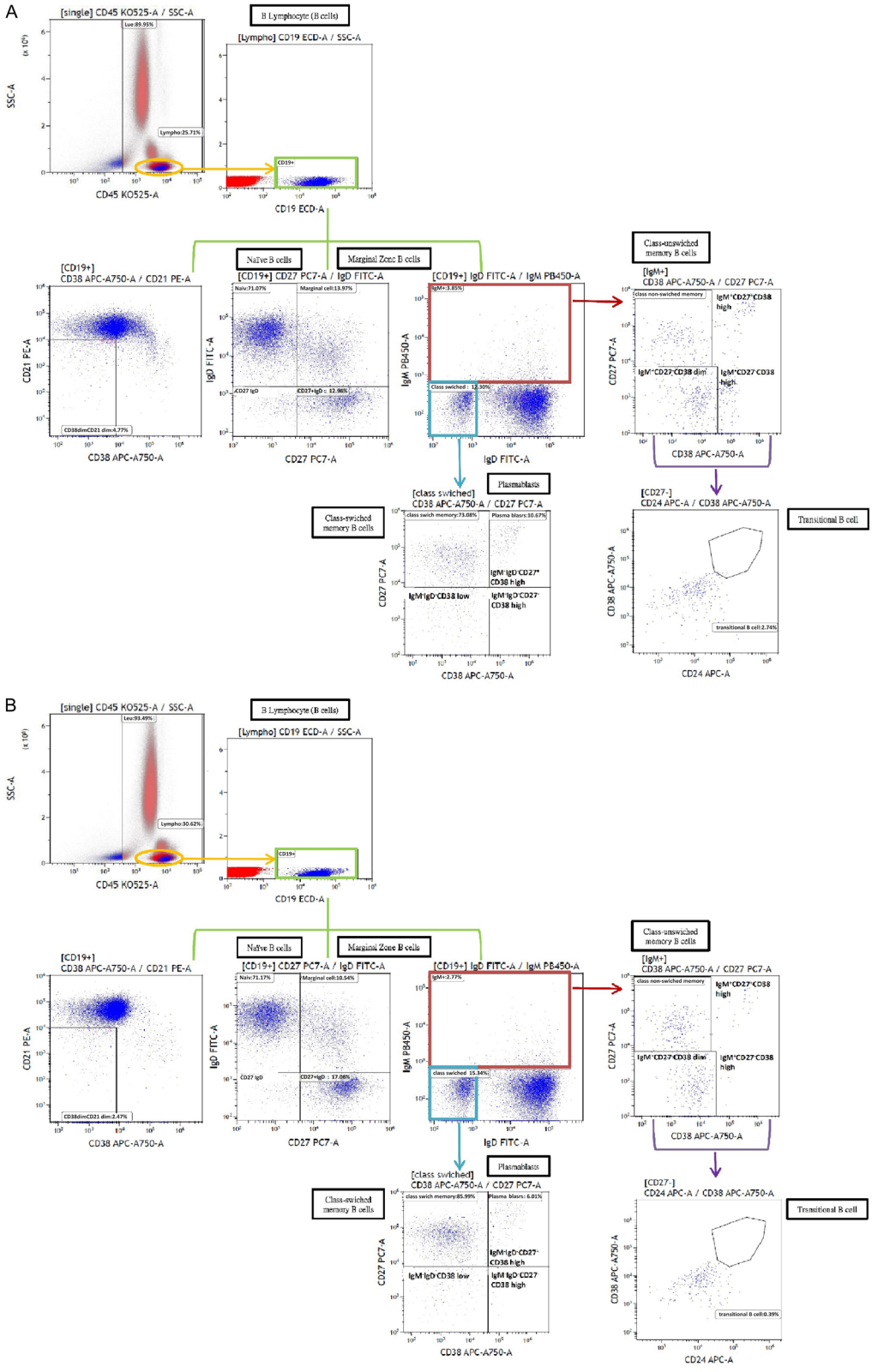
The numeric data and proportions of cell types and cytokines were presented as mean ± standard deviation. ANOVA with post-hoc comparison was used to compare the proportion of each cell subtype and the level of cytokines between the control group, early-stage, and advanced-stage patients.

For the advanced-stage cancer patients, survival analysis was performed. Survival was defined as the time from treatment to death. All subtypes of leukocytes were divided into high-frequency and low-frequency groups using the median as the cut-off point. The log-rank test was used, and survival curves were plotted using Kaplan-Meier methods, followed by multivariate analysis using COX regression. The relationship between the frequency of cell subtypes and the level of cytokines was analyzed using linear correlation, and p-values < 0.05 were considered significant.

Result

Clinicopathologic characteristics of the lung cancer patients

A total of 60 lung cancer patients were examined in this study. Out of these patients, 31 (53%) were diagnosed at an early stage (Stage I and II), while 29 (47%) were diagnosed at an advanced stage (Stage III and IV). Adenocarcinoma was the predominant type of cancer in both early-stage (81%) and advanced-stage (69%) patient groups. Surgical resection was the primary treatment option for early-stage patients (87%), while all advanced-stage patients (100%) received systemic therapy such as immunotherapy, target therapy, or chemotherapy. There was no recurrence of cancer observed in the early-stage group, whereas the median survival time for the advanced-stage group was 407 (IQR = 196-628) days (**Table 1**).


A
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B Lymphocyte (B cells)
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Naïve B cells
Marginal Zone B cells
Class-unswiched memory B cells
Class-swiched memory B cells
Plasmablasts
Transitional B cell
B
[single] CD45 KO525-A / SSC-A
B Lymphocyte (B cells)
[Lympho] CD19 ECD-A / SSC-A
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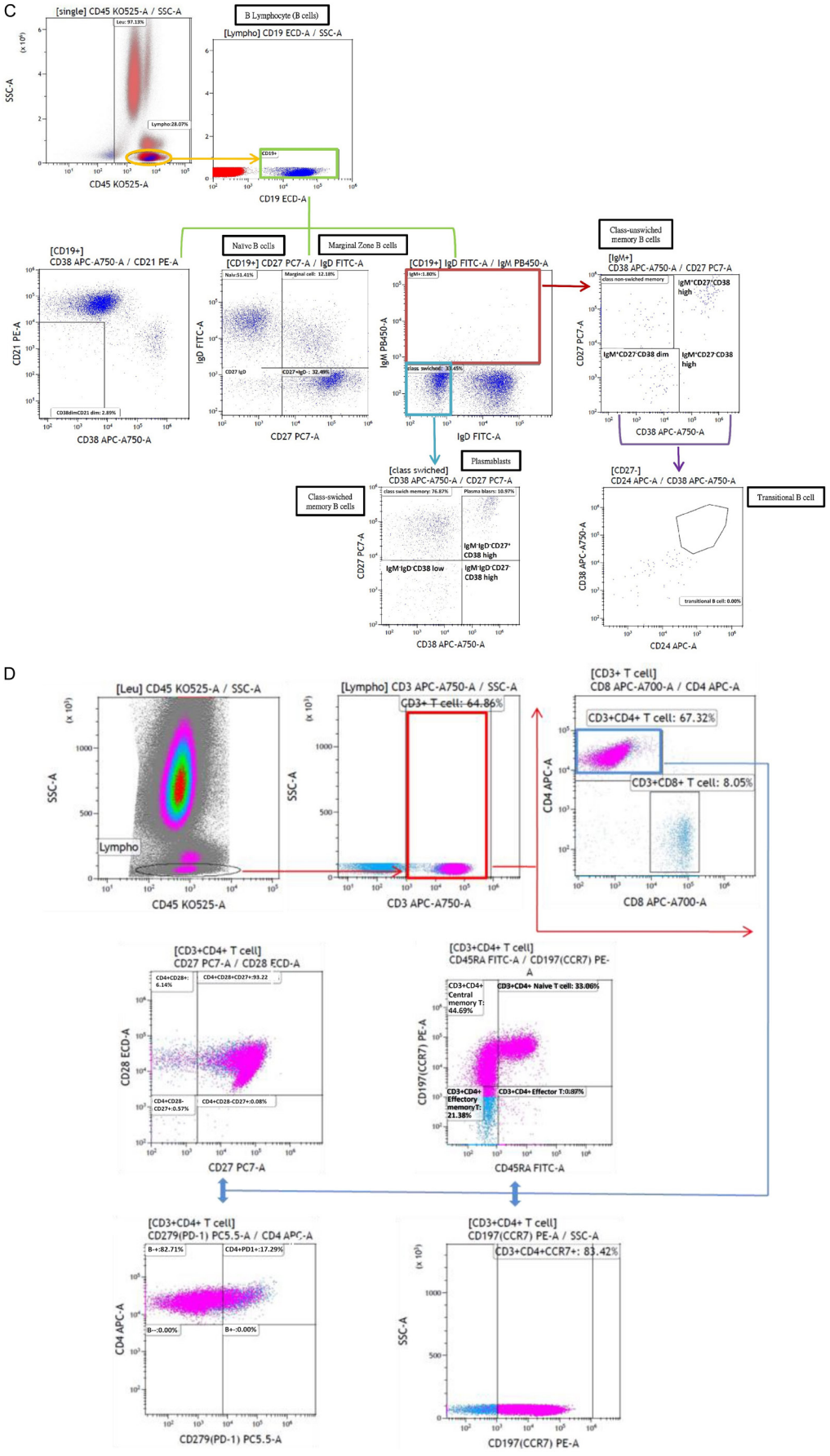
C
[single] CD45 KO525-A / SSC-A
B Lymphocyte (B cells)
[Lympho] CD19 ECD-A / SSC-A
CD45 KO525-A
CD19 ECD-A
Naive B cells
Marginal Zone B cells
[CD19+] CD38 APC-A750-A / CD21 PE-A
[CD19+] CD27 PC7-A / IgD FITC-A
[CD19+] IgD FITC-A / IgM PB450-A
Class-unswiched memory B cells
[IgM+] CD38 APC-A750-A / CD27 PC7-A
Plasmablasts
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Class-swiched memory B cells
[CD27-] CD24 APC-A / CD38 APC-A750-A
Transitional B cell
D
[Leu] CD45 KO525-A / SSC-A
Lympho
[Lympho] CD3 APC-A750-A / SSC-A
CD3+ T cell: 64.86%
[CD3+ T cell] CD8 APC-A700-A / CD4 APC-A
CD3+CD4+ T cell: 67.32%
CD3+CD8+ T cell: 8.05%
[CD3+CD4+ T cell] CD27 PC7-A / CD28 ECD-A
[CD3+CD4+ T cell] CD45RA FITC-A / CD197(CCR7) PE-A
CD3+CD4+ Naive T cell: 33.06%
[CD3+CD4+ T cell] CD279(PD-1) PC5.5-A / CD4 APC-A
[CD3+CD4+ T cell] CD197(CCR7) PE-A / SSC-A
CD3+CD4+CCR7+: 83.42%

Figure 1. Flow cytometry analysis of B cell and T cell sub-population in non-small cell lung cancer (NSCLC) patients. The frequency and characteristics of CD19+ B cells, including their antigen experience, activation, and class-switching status, were analyzed using flow cytometry with a 10-color Navios Flow Cytometer (Beckman Coulter). Various B cell subsets, such as naive, switched memory, unswitched memory, atypical memory, and marginal zone-like B cells, were identified based on the frequencies of CD27, IgM, and IgD expression. Patients were: (A) Normal healthy control (No. 3); (B) Early stage NSCLC (patient No. 102); (C) Advanced stage NSCLC (patient No. 113). The DuraClone IM T Cell Subset dry reagent kit was used to identify cell compartments in human whole blood samples with a 13 color/3 laser CytoFLEX Flow Cytometer (Beckman Coulter). Patients were: (D) Advanced stage NSCLC (patient No. 113).

Table 1. The clinicopathologic characteristics of both the normal health control group and the lung cancer patients

	Control (n = 6)	NSCLC stage I-II (n = 31)	NSCLC stage III-IV (n = 29)
Age (median)	43	58	68
Male	50%	29%	59%
Smoking	0%	19%	48%
Pathology			
AIS/MIA		5 (16%)	
Adenocarcinoma		25 (81%)	20 (69%)
Squamous cell carcinoma		1 (3%)	7 (24%)
LELC			1 (3%)
Pleomorphic carcinoma			1 (3%)
Treatment			
Surgical resection		27 (87%)	
Surgical resection with adjuvant therapy		4 (13%)	4 (13%)
Target therapy			3 (10%)
CCRT			6 (21%)
Chemotherapy			9 (31%)
Palliative			5 (17%)
Prognosis		No recurrence	
Survival (median, days)			497 (196-628)

NSCLC: non-small cell lung cancer; AIS: adenocarcinoma in situ; MIA: minimally invasion adenocarcinoma; LELC: lymphoepithelioma-like carcinoma; CCRT: concurrent radiochemotherapy.

The difference of B cells and T cell subpopulations between control group and cancer patients

The frequencies of B cell subsets were compared between a control group (n = 6) and a group of cancer patients. The control group demonstrated significantly higher frequencies of IgD+CD27+ marginal B cells (14.4 ± 3.9% vs. 8.5 ± 5.9%, $P = 0.020$) and CD32+ B cells (27.8 ± 16.9% vs. 16.4 ± 10.0%, $P = 0.016$) than the cancer group (**Table 2**).

The study evaluated the percentage of T cells in the lymphocyte stage in both cancer patients and a control group. The results showed that the frequency of T cells was different between the two groups. Specifically, cancer patients had a lower frequency of CD8+ T cells, but higher frequencies of central memory and naïve CD4+ T cells and lower frequencies of effector memory CD4+ T cells. The frequency of effector CD8+ T cells was higher, but the frequency of effector memory CD8+ T cells was lower in cancer patients. The analysis of CD27 and CD28 expression on T cells showed that cancer patients had a higher proportion of CD27+CD28+ CD4+/CD8+ T cells but a lower proportion of CD27-CD28- CD4+/CD8+ T cells, as well as lower CD27+CD28- CD4+/CD8+ T cells compared to the control group. Additionally, the expression of CD57 on CD4+ and CD8+ T cells was higher in cancer patients than in the control group (**Table 3**).

Table 2. The difference of B cell sub-population (mean ± standard deviation) between control group and cancer patients

	Normal healthy control (n = 6)	Lung cancer patients (n = 60)	*p* value
Leucocyte (%)	88.5 ± 14.3	82.2 ± 19.0	0.595
Lymphocyte (%)	35.0 ± 10.8	25.2 ± 12.7	0.071
B cell			
CD38dimCD21dim	4.7 ± 2.4	4.1 ± 4.5	0.736
Naïve (IgD+CD27-)	66.6 ± 10.0	66.4 ± 17.9	0.977
Marginal B cell (IgD+CD27+)	14.4 ± 3.9	8.5 ± 5.9	0.020*
CD27+IgD-	15.0 ± 4.8	20.3 ± 11.7	0.282
IgM+	2.9 ± 1.2	2.4 ± 1.9	0.479
Class-switched	16.7 ± 7.2	18.8 ± 11.5	0.672
Class-switched memory	73.2 ± 5.5	65.1 ± 16.6	0.240
Plasmablast	5.3 ± 3.7	13.1 ± 16.0	0.238
Transitional B cell	2.8 ± 0.9	1.9 ± 3.5	0.538
CD32+CD19+	27.8 ± 16.9	16.4 ± 10.0	0.016*
CD19+	72.2 ± 16.9	82.4 ± 13.6	0.089

*$P < 0.05$.

Table 3. The difference of T cell sub-population (mean ± standard deviation) between control group and cancer patients

	Normal healthy control (n = 6)	Lung cancer patients (n = 60)	*p* value
Leucocyte (%) (T cell)	81.70 ± 13.68	93.97 ± 10.67	0.011*
CD3+ T cell	7.09 ± 3.37	56.07 ± 16.13	0.000***
CD3+CD4+ T cell	44.11 ± 8.32	55.06 ± 12.53	0.041*
CD3+CD8+ T cell	31.45 ± 10.54	19.74 ± 7.34	0.001**
CD3+CD4+ Central memory T cell	34.02 ± 13.59	48.17 ± 10.91	0.004**
CD3+CD4+ Naïve T cell	6.46 ± 6.09	28.78 ± 15.37	0.001*
CD3+CD4+ Effector memory T cell	59.07 ± 12.27	21.65 ± 13.31	0.000***
CD3+CD4+ Effector T cell	0.46 ± 0.17	1.40 ± 2.96	0.439
CD3+CD8+ Central memory T cell	6.97 ± 4.38	12.56 ± 7.53	0.079
CD3+CD8+ Naïve T cell	21.77 ± 13.27	22.97 ± 14.35	0.844
CD3+CD8+ Effector memory T cell	52.24 ± 19.16	29.43 ± 14.85	0.001**
CD3+CD8+ Effector T cell	19.02 ± 13.93	35.04 ± 15.66	0.019*
CD4+CD28+	15.19 ± 4.95	9.10 ± 4.51	0.003**
CD4+CD28+CD27+	32.13 ± 12.93	81.65 ± 18.62	0.000***
CD4+CD28-CD27-	10.47 ± 5.19	4.46 ± 5.04	0.007**
CD4+CD28-CD27+	42.21 ± 18.05	4.79 ± 15.68	0.000***
CD8+CD28+	2.322 ± 1.65	3.53 ± 2.52	0.258
CD8+CD28+CD27+	9.52 ± 6.90	50.34 ± 20.90	0.000*
CD8+CD28-CD27-	46.98 ± 12.11	34.11 ± 16.45	0.067
CD8+CD28-CD27+	41.18 ± 13.06	12.03 ± 9.90	0.000*
CD4+PD1+	11.09 ± 5.62	19.23 ± 8.82	0.031*
CD8+PD1+	12.13 ± 3.11	26.83 ± 17.91	0.050
CD4+CD57+	3.95 ± 2.53	23.12 ± 18.06	0.012*
CD8+CD57+	32.10 ± 10.96	48.39 ± 20.31	0.058
CD3+CD4+CD45RA+	7.80 ± 6.38	29.86 ± 15.46	0.001**
CD3+CD4+CCR7+	41.10 ± 12.38	82.23 ± 14.18	0.000***

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

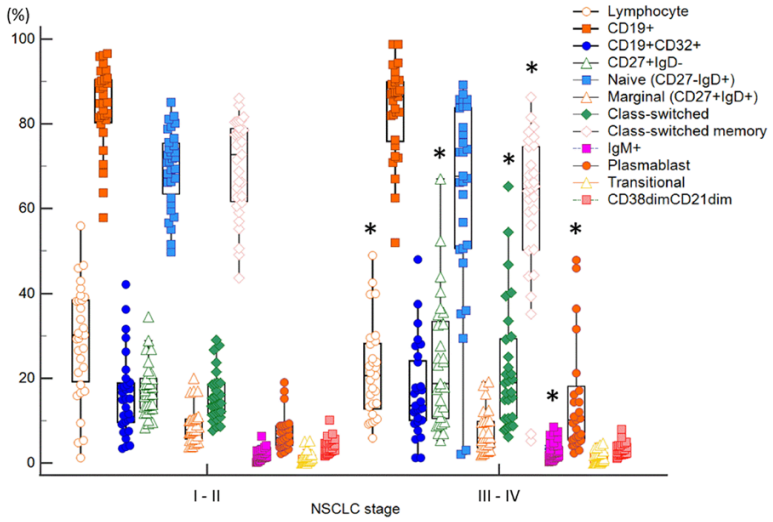

Figure 2. The difference of B cell sub-population between early-stage (I-II) and advanced-stage (III-IV) of non-small cell lung cancer (NSCLC) patients. The box plot displays the 25th to 75th percentile range of each B cell sub-population, while the line indicates the mean value along with its corresponding standard deviation. The statistical analysis showed that the frequencies of B cell subsets differed significantly (*P < 0.05) between early-stage and advanced-stage lung cancer patients.

The difference of immune cells subgroups between early stage and advanced stage lung cancer patient

When comparing the frequencies of B cell subsets between early-stage and advanced-stage lung cancer patients, several subtypes of B cells were found to have significant differences, including memory B cell, CD27+IgM+ B cell, class-switched B cell, class-switched memory B cell, and plasmablast (**Figure 2**). Linear regression analysis was performed to examine the correlation between the frequency of B cell subtypes and cancer stage, and the results showed a significant correlation between cancer stage and the frequency of B cell, memory B cell, class-switched B cell, and plasmablast after excluding the control group (coef. -2.702, P = 0.028; coef. +2.591, P = 0.023; coef. +2.813, P = 0.012; coef. +3.236, P = 0.039, respectively).

Advanced-stage lung cancer patients exhibited a higher frequency of effector memory CD8+ T cells and a lower frequency of naïve CD8+ T cells, while the frequency of CD27+CD28+ CD4+/CD8+ T cells was reduced in the advanced-stage cancer groups. Conversely, the frequency of CD28-CD28-CD8+ T cells was increased in the advanced-stage cancer group. The expression of PD-1 on T cells was not found to differ between stages. A linear regression model was employed to investigate the correlation between cancer stage and the frequency of T cell subpopulations, revealing significant correlations between cancer stage and naïve CD8+ T cells (coef. -3.282, P = 0.019), CD27+ CD28+CD4+ T cells (coef. -4.314, P = 0.017), CD27+ CD28-CD4+ T cells (coef. 3.846, P = 0.012), CD27+ CD28+CD8+ T cells (coef. -6.857, P = 0.001), CD27+ CD28-CD8+ T cells (coef. 2.124, P = 0.028), and CD27- CD28-CD8+ T cells (coef. 2.134, P = 0.028).

The total leukocyte count and proportion of neutrophils increased with advancing cancer stage, while the proportion of lymphocytes decreased (Supplementary Table 2). The levels of IL-1B, IL-4, IL-6, IL-8, IL-10, IL-12, and IL-13, IL-17, TNF-α, and TSLP were significantly higher in advanced-stage (stage III-IV) cancer patients (n = 38) compared to the early-stage NSCLC (n = 36) (stage III-IV, vs. stage I-II, mean ± SD: IL-1B, 18.27 ± 21.73, vs. 6.84 ± 6.57, P = 0.003; IL-4, 7.86 ± 10.26, vs. 2.85 ± 4.52, P = 0.09; IL-6, 47.54 ± 52.94, vs. 7.31 ± 6.42, P < 0.001; IL-8, 13.33 ± 16.46, vs. 4.24 ± 6.18, P = 0.003; IL-10, 12.96 ± 14.02, vs. 3.96 ± 6.35, P = 0.001; IL-12, 17.83 ± 20.00, vs. 8.28 ± 10.77, P = 0.013; IL-13, 1.71 ± 1.97, vs. 2.42 ± 6.96, P = 0.001; IL-17, 15.67 ± 19.86, vs. 6.51 ± 8.07, P = 0.012; TNF-α, 16.21 ± 21.83, vs. 7.19 ± 7.40, P = 0.021; and TSLP, 11.82 ± 13.72, vs. 5.83 ± 8.34, P = 0.027) (**Figure 3**). However, there was no significant correlation between the levels of IL-5, and TGF-β with cancer stage.

The prognostic value of each biomarker in the advanced-stage lung cancer patients

As no recurrent events were observed in the early-stage lung cancer cohort, we conducted survival analysis on the advanced-stage lung cancer patients. The patients were stratified into higher and lower groups based on the

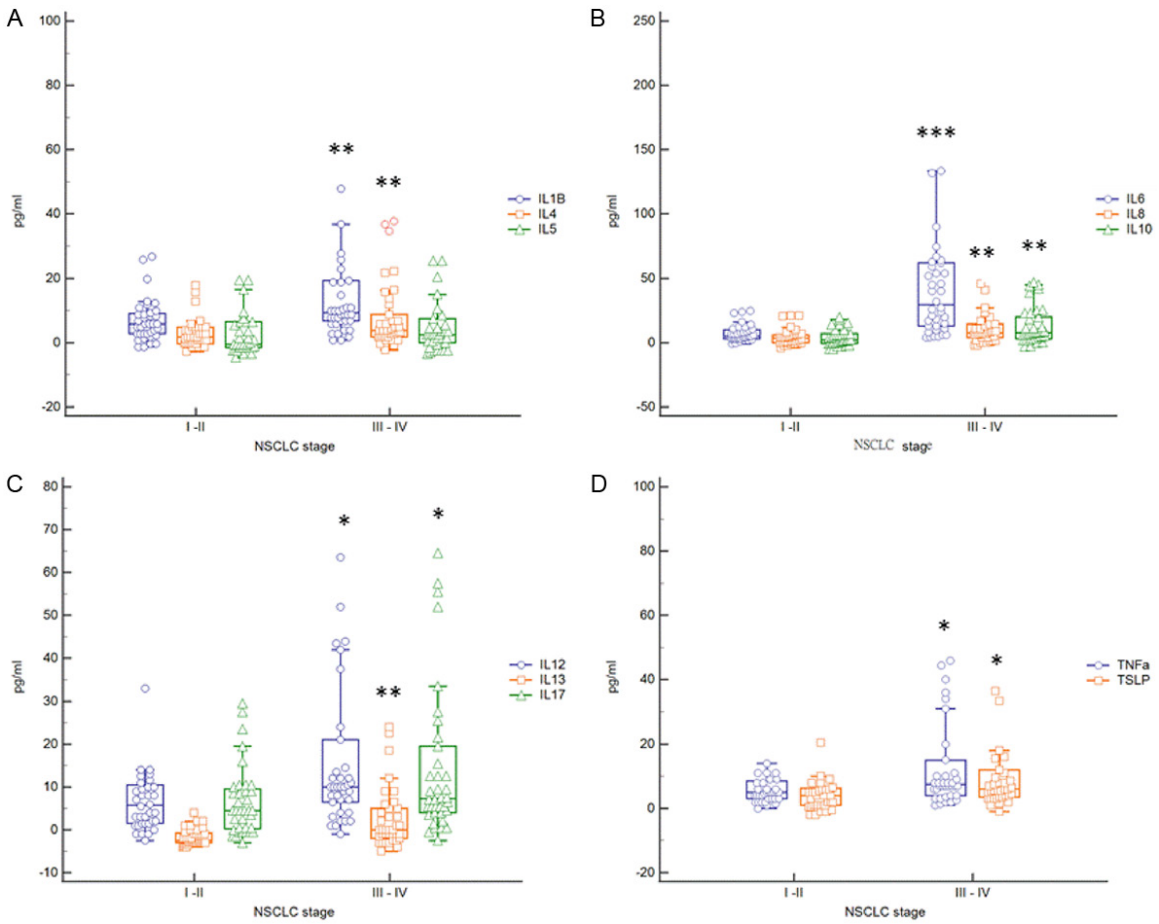

Figure 3. Cytokine expression profiles at different stages of non-small cell lung cancer (NSCLC) patients. A. Expression levels of Interleukin-1b (IL-1b), IL-4, and IL-5; B. Expression levels of Interleukin-6 (IL-6), IL-8, and IL-10; C. Expression levels of Interleukin-10 (IL-10), IL-12, IL-13, and IL-17; D. Expression levels of Tumor Necrosis Factor-α (TNF-α) and thymic stromal lymphopoietin (TSLP), depicted as a line graphs. The line represents the mean expression and one standard deviation from the mean. The box plot represents the distribution of expression within the 25th to 75th percentile range. Statistical significance is denoted as follows: *P < 0.05, **P < 0.01, ***P < 0.001.

median cut-off point for each biomarker, which included the frequency of T and B cell subsets, leukocyte count, and cytokine level. Kaplan-Meier curves and log-rank test *p* values for each biomarker are presented (**Figure 4**, Supplementary Figures 1 and 2).

Patients with a higher frequency of class-switched B cells had a significantly worse prognosis than those with a lower frequency (hazard ratio [HR]: 3.054, 95% confidence interval [CI]: 1.007-9.262, *P* = 0.049) (**Figure 4**). Although statistically insignificant, patients exhibiting elevated levels of CD4+ effector memory T cells, CD8+ effector memory T cells, CD8+ effector T cells, CD4+CD28+CD27+ T cells, CD3+CD4+CCR7+ T cells, and diminished levels of CD8+PD1+ and CD4+57+ T cells, alongside an increased ratio of CD38dimCD21dim B cells and decreased expression of marginal and class-switched memory B cells, as well as heightened concentrations of IL-8, IL-5, and TSLP, appeared to demonstrate improved survival rates. Conversely, no significant disparity in survival was discerned based on leukocyte subset levels. Remarkably, multivariate Cox regression analysis identified a higher proportion of class-switched B cells as an independent factor associated with diminished survival in NSCLC patients (HR: 2.272, 95% CI: 1.339-6.897, P = 0.044).

The correlation between the class-switched B cell and T cells

Linear regression was employed to investigate the relationship between the frequency of class-switched B cells and various T cell sub-

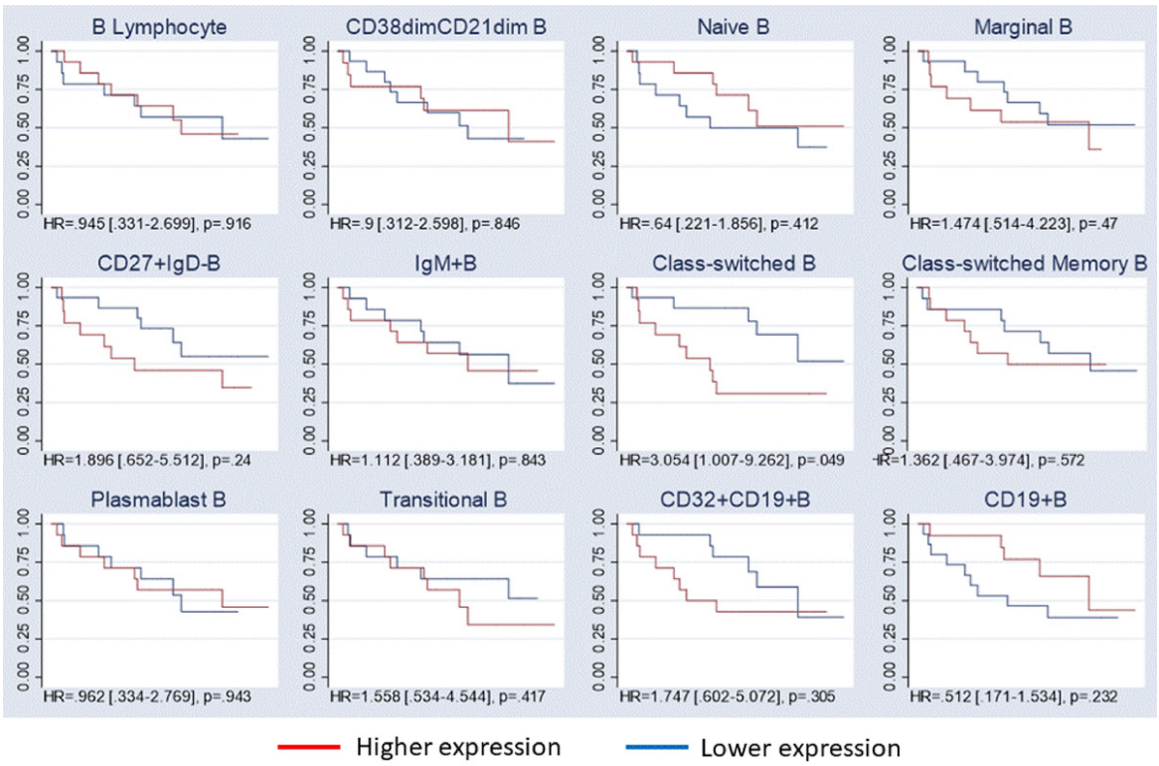

Figure 4. The Kaplan-Meier survival curve analysis for B cell sub-populations in advanced non-small cell lung cancer (NSCLC). The expression levels of each B cell sub-population were dichotomized using the median value as the cut-off.

types. The frequency of class-switched B cells was significantly correlated with the frequency of CD4+ effector memory T cells (coef. = +0.379, P = 0.011), CD8+ effector memory T cells (coef. = +0.394, P = 0.017), CD4+CD27+CD28+ T cells (coef. = -0.547, P = 0.008), CD4+CD27+CD28- T cells (coef. = 0.441, P = 0.012), CD8+CD28+ T cells (coef. = 0.062, P = 0.030), and CD4+CCR7+ T cells (coef. = -0.430, P = 0.006). The B cell subtypes are significantly correlated with each T cell subtype, however, there was no significant correlation between the frequency of class-switched B cells and the levels of serum cytokines (Supplementary Table 3).

Discussion

This study examined immune profiles in lung cancer patients, comparing early and advanced-stage cases. Differences were found in various B cell and T cell subtypes between the stages. Advanced-stage patients had more effector memory CD8+ T cells and fewer naive CD8+ T cells. The study also revealed associations between cancer stage and certain B and T cell subtypes. Additionally, certain cytokines were elevated in advanced-stage patients. The study indicated that higher class-switched B cell frequency correlated with worse prognosis, while more CD8+ effector T cells and fewer CD4+57+ T cells were associated with better survival. This suggests potential for personalized immune-focused therapies to enhance patient outcomes.

In this study, we comprehensively analyzed T and B cell markers along with cytokine levels in NSCLC patients, aiming to assess their correlations and impact on patient survival. Lymphocytes were identified using CD45 as a gating marker along with side scatter properties, while T cell subsets were delineated using markers such as CD3, CD4, and CD8, allowing for the distinction between T helper and cytotoxic T cells. Additional markers like CD45RA, CCR7, CD27, CD28, CD57, and PD-1 further characterized T cell populations, aiding in identifying subsets such as naïve, effector memory, and exhausted T cells [26]. Concurrently, B cells from peripheral blood in NSCLC patients were analyzed, with subsets categorized based

on IgD, CD24, and CD27 expression, revealing differences in distribution compared to blood and lymph nodes. Memory B cells predominated in tumors, while naive B cells were more abundant in blood and lymph nodes. This skew towards memory B cells in NSCLC patients suggests a potential role in determining protective T cell responses and serves as a novel prognostic biomarker for patient survival [13, 16, 27]. Furthermore, we investigated the role of cytokines in the TME of NSCLC, highlighting their complex and often antagonistic roles in immune maturation, host defense, and tumor progression. Various cytokine families, including interleukins (ILs), TGFβ, and TNFα, were examined, shedding light on their involvement in tumor growth and metastasis within the lung TME [28, 29].

In this study, we observed an increase in the proportion of IgM+ B cells, plasmablasts, and class-switched B cells in advanced-stage lung cancer patients. However, there was a decrease in the proportion of class-switched memory B cells in these patients. These findings suggest that B cell responses are activated in patients with lung cancer [30], which underscores the importance of examining B cell receptor (BCR) clonality and diversity in patients undergoing cancer therapy [31]. It is worth noting that the peripheral immune cell phenotype was studied in only one study, and therefore, more research is needed to validate these findings. In a healthy population, B cells account for 11.3% of lymphocytes, with the subpopulations consisting of naïve B cells (54%), memory B cells (21%), class-switched memory B cells (16%), class-unswitched B cells (6%), marginal B cells (6%), plasmablasts (1%), and transitional B cells (6%) [32]. A flow cytometry study examining tumor-infiltrating B cells in non-small cell lung cancer (NSCLC) showed that the composition included naïve B cells (1.6% of leukocytes), CD27+CD38+/- B cells (4.2%), plasma cells (0.8%), and IgM+IgD- B cells (2.2%). Furthermore, the proportion of naïve B cells decreased as the lung cancer stage progressed [33].

Our findings indicate that an increase in peripheral class-switched B cells (CD27+CD38+/-) is associated with poor survival in advanced-stage lung cancer patients. However, compared to T cells, there are fewer studies on the prognostic value of peripheral B cell subtypes in lung cancer. In NSCLC patients, the frequency of peripheral Treg, naïve CD4+ T, and naïve CD8+ T cells is correlated with overall survival [34]. Studies on the clinical prognostic value of B cell subtyping have primarily focused on tumor-infiltrating B cells. In one study characterizing NSCLC at the molecular level, the gene signature of tumor-infiltrating B cells of the naïve, memory, and plasma cell subtypes showed a strong trend towards favorable outcomes in lung adenocarcinoma [35]. Another study, which analyzed single-cell transcriptomes and antigen-immunoglobin in tumor-infiltrating B cells, identified two major subtypes: naïve-like B cells and plasma-like B cells. The study found that the frequency of naïve-like B cells decreases as NSCLC progresses, and their presence is significantly associated with poor prognosis. The study also demonstrated that plasma-like B cells have anti-tumor activity in early-stage NSCLC but protumor activity in advanced-stage NSCLC [36]. Overall, while there is limited research on the prognostic value of peripheral B cell subtypes in lung cancer, our study suggests that an increase in peripheral class-switched B cells (CD27+ CD38+/-) is a poor prognostic factor for survival in advanced-stage lung cancer patients.

Recent advancements in B-cell analysis have elucidated that adult circulating B cells can be categorized into three distinct subpopulations based on the expression of CD27 and IgD: IgD+CD27- naive B cells (referred to as circulating B cell 1 or cB1), IgD+CD27+ B cells (cB2), and IgD-CD27+ memory B cells (cB3). Previous research has indicated that IgM+IgD+CD27+ B cells carry somatic hypermutation, suggesting a memory cell phenotype. Furthermore, cB2 likely represent unclass-switched memory B cells with somatically mutated V-region genes, playing a crucial role in secondary immune responses by generating high-affinity IgM and IgG antibodies [37]. Interestingly, the presence of CD27-IgD- double-negative B cells has been observed in human NSCLC [38]. The therapeutic efficacy of many antitumor immunoglobulin Gs (IgGs) hinges on their interaction with Fcγ receptors (FcγRs), which plays a pivotal role in inducing tumor cytotoxicity through processes like antibody-dependent cellular cytotoxicity (ADCC). This mechanism involves Type I FcγR-expressing effector cells, including natural killer cells and macrophages [39]. Conversely, FCGR2B (CD32B) is a receptor found on B cell membranes that delivers inhibitory signals cru-

cial for immune system homeostasis, preventing B cell activation against self-antigens [40]. Thus, activating FCGR2B presents a promising therapeutic strategy for managing autoimmune and inflammatory diseases.

Our study found that cancer patients had decreased CD32+CD19+ B cells and marginal B cells compared to healthy individuals. CD32b, also known as FcγRIIb, is the only known inhibitory Fc receptor expressed on various immune cells, including dendritic cells, macrophages, neutrophils, and B cells. CD32b's inhibitory capacity depends on the expression of an intracellular ITIM, which, when phosphorylated, recruits the phosphatase SHIP1. In B cells, SHIP1 recruitment leads to reduced downstream signaling of the BCR, ultimately resulting in decreased BCR-dependent cell activation and antibody production [41]. Previous studies have shown that blocking CD32 can promote dendritic cell maturation [42], increase tumor-infiltrating CD8+ T cells in melanoma [43], and be used to treat B cell acute lymphocytic leukemia, B cell lymphoma, and multiple myeloma with anti-CD32 monoclonal antibodies [44-46]. Overall, our findings suggest that decreased CD32+CD19+ B cells and marginal B cells in cancer patients may have implications for the use of CD32-targeting therapies in cancer treatment.

In the spleen and other secondary lymphoid organs, two main types of B cells exist - follicular B cells and marginal zone B cells. Follicular B cells constitute the majority of peripheral B cells and are known to respond to protein antigens. Marginal zone B cells, on the other hand, are a distinct population of presumably naive B cells that reside in the spleen's marginal zones, where the white pulp meets the red pulp. Both types of cells originate from a common lineage at the transitional 2 stage and are poised to rapidly respond to antigens of pathogens [47]. Marginal zone B cells express high levels of CD21 and CD1d and respond vigorously to blood-borne pathogens. These cells are thought to be a significant source of lipid-specific antibodies as their high levels of CD1d facilitate the presentation of lipid antigens to invariant natural killer T (iNKT) cells. Additionally, when injected immune complexes enter the body, they rapidly bind to marginal zone B cells, which can transport them from the circulation to the splenic follicles. The high levels of CD21 on marginal zone B cells help in the presentation of immune complexes to follicular dendritic cells, which can then present them to follicular B cells [48]. Overall, marginal zone B cells and follicular B cells play critical roles in the immune response to pathogens and act together to generate an effective immune response.

Our study uncovered significant correlations between class-switched B cells and various T cell types in lung cancer. Positive associations were found with CD4+ effector memory T cells, CD8+ effector memory T cells, and CD4+CD27+CD28- T cells. Conversely, a negative correlation was observed with CD4+ CD27+CD28+ T cells. We also noted a weak positive link with CD8+CD28+ T cells and CD4+CCR7+ T cells. These findings suggest a complex interplay between B and T cells in the immune response to lung cancer. B and T cells both hold crucial roles in combatting cancer. CD8+ T cells, in particular, are vital for identifying and eliminating cancer cells. B cells can either directly present tumor-associated antigens to T cells or produce antibodies that enhance antigen presentation and combat tumor cells [49]. While B cells have been found to have protective roles by producing antibodies against tumor antigens, they can also promote tumor growth by interacting with T cells to suppress the anti-tumor immune response and secreting factors that facilitate tumor growth and metastasis [49]. The relationship between B and T cells in lung cancer is multifaceted, with potential for both beneficial and harmful effects [50]. Further research is needed to understand these interactions and develop new immunotherapeutic strategies targeting these cells.

To produce antibodies, B cells must differentiate into specific antibody-secreting cells known as plasma cells. This differentiation process is triggered by the binding of antigens to the B cell immunoglobulin receptors, as well as by the presence of cytokines such as IL-2, IL-6, and IL-10, and direct cell-to-cell contact between T and B cells [51]. However, the exact mechanism by which mature B cells differentiate into either memory B cells or plasma cells has remained unclear. Recent research has shown that CD27 is a memory marker of B cells and plays a role in the differentiation of B cells into either plasma cells or memory B cells. CD27+ B cells differentiate into plasma cells

through contact with CD27 ligand (CD70) transfectants, in cooperation with stimuli such as IL-10 [52]. This discovery sheds new light on the differentiation process of B cells and provides a potential target for the development of new immunotherapeutic strategies. Overall, the differentiation of B cells into plasma cells or memory B cells is a complex process that involves a variety of signals and interactions with other cells. Understanding the mechanisms involved in this process is essential for the development of effective treatments for a wide range of immune-related diseases.

There were several limitations that need to be considered in our study. One critical aspect for developing biomarkers of response to therapy and assessing patient response is the ability to precisely and reproducibly understand the immune cell phenotype and activation status, known as the cellular immunome, in tumor and blood [31]. Age has a significant impact on the distribution of immune cell subsets, however, we did not correct for age-related changes in immune cell subsets. Naive CD4+ and CD8+ T cells decreased with age, while memory T cells, including central memory CD4+ and terminal differentiated effector memory CD8+ T cells, increased. Additionally, there were changes in T cell subsets representing an activated/differentiated phenotype (HLADR+, CD27-, CD28-, CD57+), with a strong age-dependent increase in both CD4+ and CD8+ T cells. The subset changing the most with age was the CD27- effector T cells. Furthermore, we observed differences in B cell proportions in different sampling locations. These differences should be considered when interpreting data from studies that sample B cells from different regions [33]. Overall, understanding these limitations is crucial in accurately interpreting and applying findings from immune cell studies.

In conclusion, the study investigated the clinicopathologic characteristics and immune profiles of lung cancer patients and found significant differences in B cell and T cell subsets between early and advanced-stage cancer patients. Advanced-stage cancer patients had a higher frequency of effector memory CD8+ T cells, a lower frequency of naive CD8+ T cells, and increased levels of certain interleukins. The study also identified a correlation between the frequency of certain B cell and T cell subtypes and cancer stage. Additionally, the study investigated the association between immune profiles and prognosis and found that patients with a higher frequency of class-switched B cells had a worse prognosis, while patients with a higher frequency of CD8+ effector T cells and lower frequency of CD4+57+ T cells had a better survival rate. The study provides important insights into the immune profiles of lung cancer patients and suggests that personalized therapies targeting specific immune subtypes may be effective in improving patient outcomes. However, more research is needed to validate these findings and further investigate the prognostic value of B cell subtypes in lung cancer.

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Peripheral blood was collected upon receipt of informed consent.

Disclosure of conflict of interest

None.

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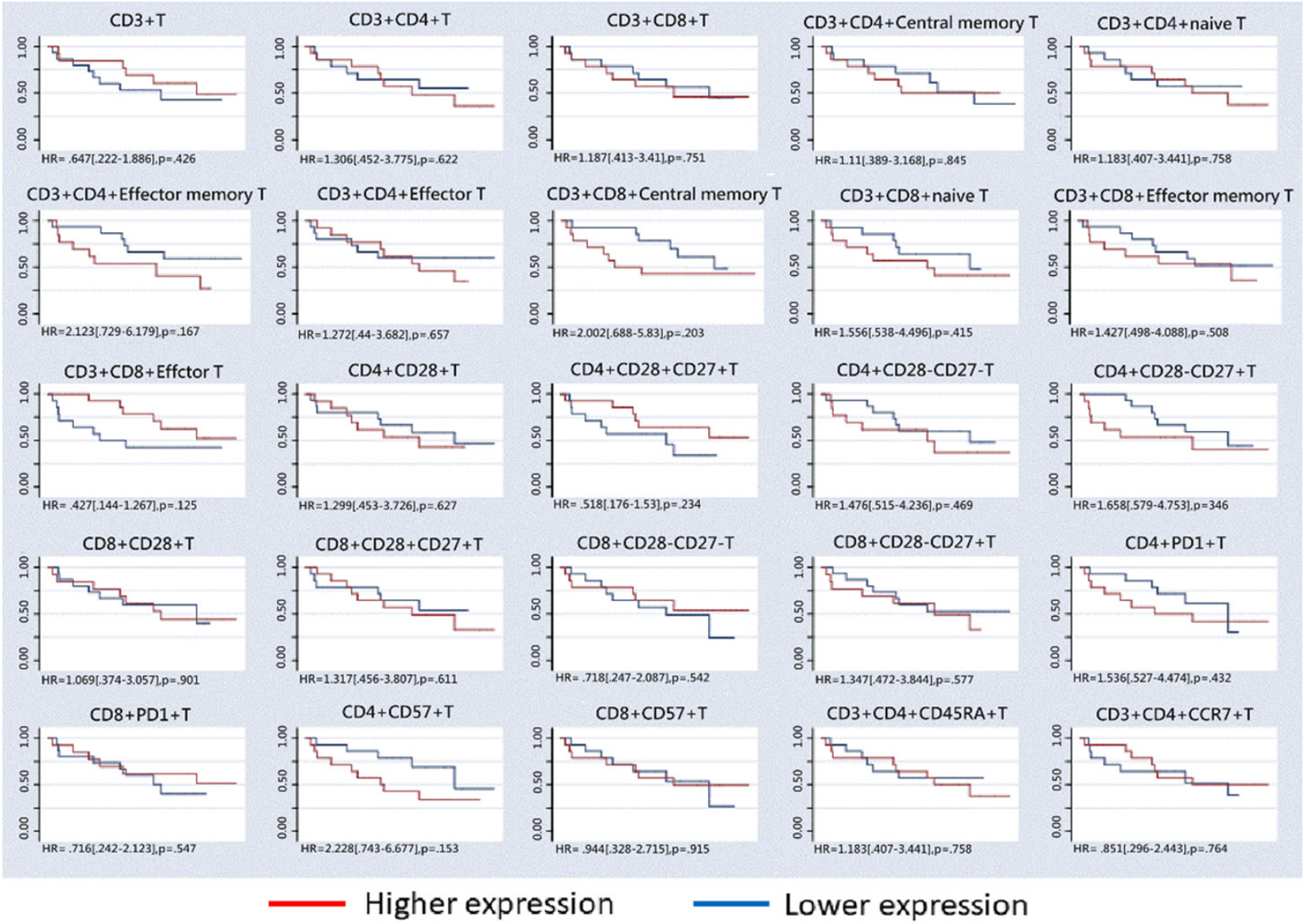
Supplementary Table 1. Basic characteristics of cytokines

Cytokine	Primary Cell Source	Primary Target Cell	Biological Activity
IL-1	Monocytes Macrophages Fibroblasts Epithelial cells Endothelial cells Astrocytes	T cells B cells Endothelial cells Hypothalamus Liver	Co-stimulation Cell activation Inflammation
IL-4	T cells	T cells B cells	Th2 differentiation Cell growth Cell activation IgE isotype switching
IL-5	T cells	B cells Eosinophils	Cell growth Cell activation
IL-6	T cells Macrophages Fibroblasts	T cells B cells Liver	Co-stimulation Cell growth Cell activation Acute phase reactant
IL-8	Macrophages Epithelial cells Platelets	Neutrophils	Activation Chemotaxis
IL-10	Th2 T cells	Macrophages T cells	Inhibits antigen-presenting cells Inhibits cytokine production
IL-12	Macrophages NK cells	T cells	Th1 differentiation
IL-13	T cells Mast cells	B cell Macrophage	Activate monocyte IgE synthesis
IL-17	NKT cells ILC	Epithelial cells Endothelial cells Fibroblasts Osteoblasts	Control of infections Initiate a potent inflammatory response
TSLP	Human epithelial cells	T cells B cells Macrophage	Activate dendritic cells Promote T helper (Th) 2 immune responses
TGF-β	T cells Macrophages	T cells	Inhibits cell growth/activation
TNF-α	Macrophages T cells	T cells B cells Endothelial cells Hypothalamus Liver	Co-stimulation Cell activation Inflammation

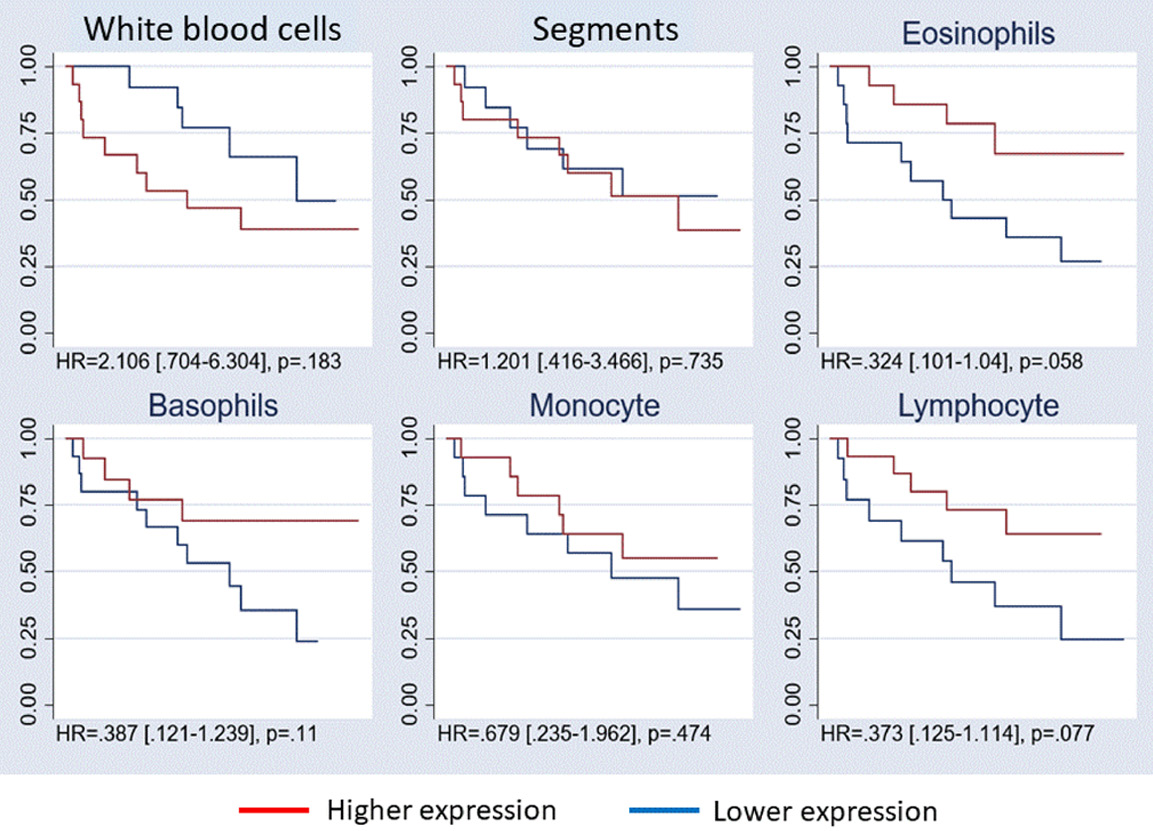
Supplementary Table 2. The difference of leucocyte subgroups between early-stage and advanced-stage lung cancer patients

	NSCLC Stage I-II	NSCLC Stage III-IV	*P* value
WBC (10^3/uL)	6.93 ± 2.88	8.62 ± 2.70	0.029
Neutrophils (%)	62.08 ± 10.51	74.31 ± 10.21	< 0.001
Eosinophil (%)	2.39 ± 1.73	1.84 ± 2.32	0.359
Basophils (%)	0.60 ± 0.38	0.43 ± 0.27	0.092
Monocyte (%)	5.96 ± 1.50	6.13 ± 2.39	0.765
Lymphocyte (%)	28.98 ± 9.77	17.29 ± 7.44	< 0.001

WBC: white blood cell.



Supplementary Figure 1. The Kaplan-Meier survival curve analysis for T cell sub-populations in advanced non-small cell lung cancer (NSCLC). The expression levels of each T cell sub-population were dichotomized using the median value as the cut-off.



Supplementary Figure 2. The Kaplan-Meier survival curve analysis for sub-populations of white blood cell in advanced non-small cell lung cancer (NSCLC). The expression levels of each white blood cell sub-population were dichotomized using the median value as the cut-off.

Supplementary Table 3. The correlation between the frequency of class-switched B cells and the levels of serum cytokines

Class-switched B cell	COEF	CI_L	CI_H	*P*_value	R2
IL1B	0.177	-0.349	0.702	0.500	0.012
IL4	0.076	-0.182	0.334	0.556	0.009
IL6	3.696	-1.346	8.737	0.146	0.055
IL8	0.140	-0.286	0.567	0.509	0.012
IL10	0.160	-0.199	0.518	0.372	0.021
IL12	0.192	-0.319	0.704	0.452	0.015
IL13	0.090	-0.089	0.269	0.314	0.027
IL17	0.182	-0.312	0.675	0.460	0.014
TNFA	0.051	-0.467	0.569	0.843	0.001
IL5	0.104	-0.266	0.473	0.574	0.008
TSLP	0.099	-0.260	0.458	0.580	0.008
TGFB	1.220	-0.462	2.902	0.150	0.054

COEF: coefficient; CI: confidence interval.