

# Elevated peripheral blood B lymphocytes and CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> T lymphocytes in patients with non-small cell lung cancer: A preliminary study on peripheral immune profile

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**Abstract.** It has been established that the tumor microenvironment (TME) has a crucial role in enabling tumors to evade from host immune responses. Previous studies demonstrated that tumor cells are not only able to reshape immune milieu at the tumor site, but also exert systemic effects, which has been demonstrated to be important for metastasis. At present, how the peripheral immune environment change in the tumor-bearing host is unclear. The present study identified a number of changes in the proportions of lymphocyte subpopulations and the levels of cytokines in patients with NSCLC, which may provide a preliminary profile of the immune environment in the peripheral blood of patients harboring a tumor. These findings expand on the present knowledge on how tumors can alter the immune system to benefit its growth and metastasis, which may provide a potential novel strategy for immunotherapy.

## Introduction

Malignant tumor is able to elicit host immune response (1-4). However, according to the cancer immunoediting theory, subpopulations of genetically heterogeneous neoplastic cells evolve to escape immune surveillance and thrive (5). During recent decades, numerous experimental and clinical studies have demonstrated that tumor cells surviving from

immune surveillance acquire various (6) capacities to change constitution and/or function of immune and stromal cells at the tumor site, creating topical immunosuppressive milieu, thus the concept of tumor microenvironment (TME) was proposed (7), which could partly explain why and how tumor cells avoid antitumor immune responses. The specific mechanisms involved in TME are highly complicated, including tumor cell exploiting co-inhibitory signaling molecules through interaction with immune cells, secretion of immunosuppressive cytokines, recruitment of immune regulatory cells [regulatory T lymphocyte (Treg) and myeloid-derived suppressor cell (MDSC)], inducing cancer-related fibroblast and tumor-associated macrophage, and other unidentified pathways (6,8-10). Notably, a number of these mechanisms are important self-protective measures from tissue damage caused by excessive immune reaction (11,12). Based on these findings, a number of monoclonal antibody agents were developed which benefit many patients with malignant tumors (13-15). To date, the critical role of TME in altering the biological behaviors of tumors have been established, and it is proposed that genetic alterations in neoplastic cells as well as the host immune system affect tumorigenesis and progression (16).

Moreover, experimental evidence suggests that primary tumor lesions are able to exert systemic effects by various means. For example, a number of studies demonstrate that the systemic effects of the tumor are able to form so-called pre-metastatic niches in distant tissues or organs, and also facilitate its own progression (17-22). By contrast, some studies indicate that the systemic effects of the tumor are able to inhibit metastasis (23,24). In spite of these inconsistent findings, it has been demonstrated that the tumor is able to exhibit systemic effects. Therefore, it is hypothesized since tumor cells are able to reshape immune and stromal cell profile at the tumor site, the systemic effects of the tumor may adapt peripheral immune components to create a pro-tumor peripheral immune environment. Nevertheless, at present, there is a lack of direct evidence for peripheral immune profile in tumor patients.

The present authors are particularly interested in tumor metastasis, which is also the most common type of relapse for the majority of malignancies following complete resection and adjuvant therapies, particularly for lung cancer, which has one of the highest cancer-associated mortalities

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**Abbreviations:** NSCLC, non-small cell lung cancer; TME, tumor microenvironment; MDSC, myeloid-derived suppressor cell; Treg, regulatory T lymphocyte; SCLC, small cell lung cancer; IFN- $\gamma$ , interferon- $\gamma$ ; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; TGF- $\beta$ , transcription growth factor- $\beta$ ; SD, standard deviation

**Key words:** non-small cell lung cancer, peripheral immune environment, metastasis, tumor, immune system

and incidence among malignant tumors according to recent statistics (25). Numerous patients with small primary malignant lesions develop metastasis, which suggests that metastasis is a relatively unique process in proportionate with the topical progression of the tumor (26).

At present, there is limited information regarding why and how tumor cells can be safely transported through the blood or lymphatic vessel to another organ or lymph node without being captured and killed by circulating immune components. Another question is how latent micrometastasis develop to be clinically detectable. Theoretically, if the peripheral immune system were robust, it would be highly probable for metastatic tumor cells in circulation to be eliminated. Therefore, the present authors hypothesize that the peripheral immune environment may be adapted in a way so the metastatic cells can evade from the immune response.

In the present study, a preliminary study was performed to characterize the profile of the peripheral circulating immune system in patients with non-small cell lung cancer (NSCLC) and healthy controls by assaying the proportion of lymphocyte subpopulations and the levels of a number of tumor-associated cytokines. Furthermore, comparisons were also performed between NSCLC patients with and without metastasis.

## Materials and methods

The present study was conducted following Human Experimentation Review and approved by Research Ethics Committee of the General Hospital of People's Liberation Army and the General Hospital of Beijing Command. Informed consent was obtained from all patients enrolled in the present study, and information from the patients was protected. From April 2015 to December 2015, 48 eligible patients with NSCLC, who were admitted to either the General Hospital of Beijing Command (Beijing, China) or the General Hospital of People's Liberation Army (Beijing, China), were included in the present study. Patient age ranged from 42-72 years, with the mean age of 56. The inclusion criteria were as follows: i) Having a clinical diagnosis of lung cancer; ii) being newly diagnosed without receiving any antitumor therapy; iii) without acute or chronic inflammatory disease during study; iv) without suffering from immunodeficiency condition; v) without suffering from immune-related disease; vi) without a history of long-term drug therapy that may affect immunity. The exclusion criteria were as follows: i) Pathological diagnosis of benign disease or small cell lung cancer (SCLC); ii) without pathological diagnosis; iii) incomplete examinations and iv) the presence of other concomitant malignancy. A total of 21 patients admitted to General Hospital of Beijing Command were also included as the control group: Two patients were pathologically diagnosed as lung hamartoma, one patient was pathologically diagnosed as costal fibrous dysplasia, and 18 patients were diagnosed as congenital chest wall deformity. The inclusion criteria for the control group were as follows: i) Without any type of malignant tumor; ii) without acute or chronic inflammatory disease during study; iii) without suffering from immunodeficiency disorders; iv) without suffering from immune-related disease and v) without a history of long-term drug therapy that may affect immunity. All participants were divided into two groups: NSCLC group and control group. Additionally, the NSCLC

group was further divided into two subgroups: Subgroup I (NSCLC at stage I; n=17) and subgroup II (NSCLC with metastasis; n=31). In subgroup I, 16 patients were at pathological stage I, and 1 patient was at clinical stage I. In subgroup II, lymph node metastasis was pathologically confirmed, and distant metastasis was confirmed with imaging.

*Peripheral blood and serum sample.* Peripheral venous blood were obtained from patients in the morning and stored separately in heparin-coated and non-coagulated tubes. The blood samples were transferred immediately to the laboratory. Serum was aliquoted by centrifugation (200 x g for 10 min) at room temperature, then stored at -80°C for subsequent assay to detect the level of cytokines.

*Flow cytometric assay of lymphocyte subpopulations.* The reagents used for immunostaining were as follows: Fluorescein isothiocyanate (FITC) or phycoerythrin cyanin (PC)7-conjugated anti-cluster of differentiation (CD)3 (20 µl; catalog no. 6607100) PC5-conjugated anti-CD4 (10 µl; catalog no. H07752), FITC-conjugated anti-CD8 (20 µl; catalog no. H07756), phycoerythrin (PE)-conjugated anti-CD25 (20 µl; catalog no. H07774), PC7-conjugated anti-CD19 (10 µl; catalog no. IM3628), PE-conjugated anti-CD56 (20 µl; catalog no. H07788) and PE-conjugated anti-CD16 (20 µl; catalog no. H07766) (all from Beckman Coulter, Inc., Brea, CA, USA).

*Cell staining.* Blood samples were stained according to the manufacturer's instructions, and the following panels were designed: i) CD8-FITC/CD25-PE/CD45-ECD/CD4-PC5/CD3-PC7 and ii) CD3-FITC/CD16<sup>+</sup>56-PE/CD45-ECD/CD14-PC5/CD19-PC7.

*Quantification by flow cytometry.* Following staining, the blood samples were assayed using a 5-colored uni-laser flow cytometer (FC500; Beckman Coulter, Inc.). Data analysis was undertaken using the CXP software (Beckman Coulter, Inc., Brea, CA, USA). The combinations of antibodies used for analysis are follows: CD3<sup>+</sup> for T lymphocytes, CD19<sup>+</sup> for B lymphocytes, CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> for double-negative T lymphocytes (DN T cells), CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> for activated T lymphocytes, CD3<sup>+</sup>CD4<sup>+</sup>CD25 high roughly for Treg, CD3<sup>+</sup>CD16<sup>+</sup>CD56<sup>+</sup> for natural killer T cells and CD3<sup>+</sup>CD16<sup>+</sup>CD56<sup>+</sup> for natural killer cells.

*Flow cytometric assay of serum cytokines.* Serum cytokine levels [including interferon (IFN)-γ, tumor necrosis factor (TNF)-α, transcription growth factor (TGF)-β, interleukin (IL)-2, IL-4, IL-6, IL-10 and IL-17A] were assayed by using the commercially available Aimplex human Th1/Th2/Th17plex assay kit and the human TGF-β assay kit, from AimPlex Biosciences, (Pomona, CA, USA) and Beijing Quantobio Biotechnology Co., Ltd. (Beijing, Chin), respectively, following the manufacturer's instructions. Quantitation measurements were performed by a 4-colored uni-laser flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA). FCAP Array software (version 3.0) was used to process data. Standard curves for each type of cytokine were generated with manufacturer-supplied reference analytes.

Table I. Basic information of participants.

| Parameters                     | NSCLC group |             | Control group |    |     |
|--------------------------------|-------------|-------------|---------------|----|-----|
|                                | Subgroup I  | Subgroup II | CCWD          | LH | CFD |
| Gender                         |             |             |               |    |     |
| Male                           | 8           | 22          | 14            |    | 1   |
| Female                         | 9           | 9           | 4             | 2  |     |
| Pathology                      |             |             |               |    |     |
| Adenocarcinoma                 | 12          | 17          |               |    |     |
| Squamous cell carcinoma        | 3           | 11          |               |    |     |
| Adenosquamous carcinoma        | 2           | 1           |               |    |     |
| Large-cell lung carcinoma      | 0           | 2           |               |    |     |
| Metastasis                     |             |             |               |    |     |
| N1-N2                          | 0           | 14          |               |    |     |
| N3 or distant organ metastasis | 0           | 17          |               |    |     |

CCWD, congenital chest wall deformity; LH, lung hamartoma; CFD, costal fibrous dysplasia; NSCLC, non-small cell lung cancer; N1, hilar or intralobal lymph node metastasis; N2, ipsilateral mediastinal lymph node metastasis; N3, contralateral hilar or mediastinal or supraclavicular lymph node metastasis.

**Statistical analysis.** The data are presented as the mean or mean  $\pm$  standard deviation. To compare the proportion of lymphocytes and subpopulations between groups, Student's t-test or Wilcoxon rank-sum test were used. Similarly, Student-test or Wilcoxon rank-sum test was used to compare concentrations of cytokines between groups.  $P < 0.05$  was considered to indicate a statistically significant difference. Statistical analysis was performed using the SPSS software (version 13; SPSS, Inc., Chicago, IL, USA).

## Results

**General characteristics of the participants.** There were 48 eligible NSCLC patients participating the present study. Among them, 30 cases were male, 18 cases were female. A total of 29 cases were diagnosed with adenocarcinoma, 14 cases with squamous cell carcinoma, 3 cases were adenosquamous carcinoma and 2 cases with large cell carcinoma (Table I).

Distribution of lymphocyte subpopulations as determined by flow cytometric analysis (Fig. 1A-D). Lymphocyte subsets between the NSCLC group and the control group were compared (Table II). The results indicated that the percentage of lymphocytes in the NSCLC group was significantly lower compared with the control group ( $P = 0.008$ ; Fig. 2). Additionally, the proportion of B cells (CD19<sup>+</sup>) among lymphocytes in the NSCLC group was significantly lower compared with the control group ( $P < 0.0001$ ; Fig. 3 and Table II). The proportion of DN T cells (CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>) among lymphocytes in the NSCLC group was significantly lower compared with the control group ( $P = 0.001$ ; Fig. 4 and Table II). However, there were no significant differences in the proportion of other subpopulations assayed (Table II). The ratio of CD4<sup>+</sup>/CD8<sup>+</sup> cells was not significantly different between the NSCLC group and the control group (Table II).

Subsequently, comparisons between subgroups I and II were performed (Table III). The percentage of lymphocytes in

subgroup I was significantly higher compared with subgroup II ( $P < 0.0001$ ; Fig. 5 and Table III). However, there were no significant differences in the proportion of other assayed subpopulations between subgroups I and II. There were also no significant differences between the two groups in the proportions of CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>high</sup> and CD3<sup>+</sup>CD4<sup>+</sup> cells (Table III).

**Levels of cytokines.** In the present study, the levels of eight types of cytokines, including IL-2, IL-4, IL-6, IL-10, IL-17A, TNF- $\alpha$ , TGF- $\beta$  and IFN- $\gamma$  were analyzed. Comparisons were performed for each of the eight cytokines between the NSCLC group and the control group. The results indicated that the levels of IL-6 in the NSCLC group were significantly higher compared with the control group (0.008) (Table IV). However, there were no significant differences for other cytokines (Table IV). Subsequently, comparisons were performed in the levels of cytokines between subgroups I and II, and no significant differences were identified (Table IV).

## Discussion

Malignant tumor remains one of the biggest threats to human health. According to statistics, the total number of cancer-associated mortalities worldwide in was 8.2 million, and the number of newly diagnosed cases was 14.1 million (25). Although intensive research has been conducted to unravel tumorigenesis and to identify novel therapeutic approaches and as a result enormous progress has been made in knowledge and clinical management, there remains to be questions regarding the underlying mechanisms of tumor.

Molecular and biological studies revealed that neoplastic cells are genetically unstable and heterogeneous, which account for complexity and diversity of tumorigenesis and its biological behaviors. Host immune response targeting malignant tumor in patients and animal models have long

Table II. Comparison of lymphocyte subsets between the NSCLC group and the control group.

| Lymphocyte subsets                                     | NSCLC group (mean $\pm$ SD) | Control group     | P-value <sup>a</sup> |
|--|-----------------------------|-------------------|----------------------|
| Lymphocyte   | 0.247 $\pm$ 0.09            | 0.318 $\pm$ 0.117 | 0.008                |
| CD3 <sup>+</sup>                                       | 0.699 $\pm$ 0.092           | 0.722 $\pm$ 0.055 | 0.288                |
| CD3 <sup>+</sup> CD4 <sup>+</sup>                      | 0.410 $\pm$ 0.08            | 0.384 $\pm$ 0.078 | 0.222                |
| CD3 <sup>+</sup> CD8 <sup>+</sup>                      | 0.267 $\pm$ 0.074           | 0.285 $\pm$ 0.062 | 0.346                |
| CD3 <sup>+</sup> CD4 <sup>+</sup> CD8 <sup>-</sup>     | 0.032 $\pm$ 0.018           | 0.054 $\pm$ 0.034 | 0.001                |
| CD3 <sup>+</sup> CD4 <sup>+</sup> CD25 <sup>+</sup>    | 0.094 $\pm$ 0.053           | 0.103 $\pm$ 0.033 | 0.076                |
| CD3 <sup>+</sup> CD4 <sup>+</sup> CD25 <sup>high</sup> | 0.002 $\pm$ 0.003           | 0.001 $\pm$ 0.002 | 0.059                |
| CD19 <sup>+</sup>                                      | 0.110 $\pm$ 0.044           | 0.152 $\pm$ 0.047 | <0.0001              |
| CD3 <sup>+</sup> CD16 <sup>+</sup> CD56 <sup>+</sup>   | 0.080 $\pm$ 0.057           | 0.081 $\pm$ 0.091 | 0.291                |
| CD3 <sup>-</sup> CD16 <sup>+</sup> CD56 <sup>+</sup>   | 0.168 $\pm$ 0.098           | 0.123 $\pm$ 0.040 | 0.080                |
| CD4 <sup>+</sup> /CD8 <sup>+</sup>                     | 1.681 $\pm$ 0.639           | 1.449 $\pm$ 0.572 | 0.159                |

Lymphocyte, percentage of lymphocytes; SD, standard deviation; CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>, double negative T lymphocyte; CD19<sup>+</sup>, B lymphocytes; CD3<sup>+</sup>CD4<sup>+</sup>CD25<sup>high</sup>, regulatory T lymphocytes; CD3<sup>+</sup>CD16<sup>+</sup>CD56<sup>+</sup>, natural killer T cells; CD3<sup>-</sup>CD16<sup>+</sup>CD56<sup>+</sup>, natural killer cells. <sup>a</sup>NSCLC group vs. control group. CD, cluster of differentiation; NSCLC, non-small cell lung cancer.

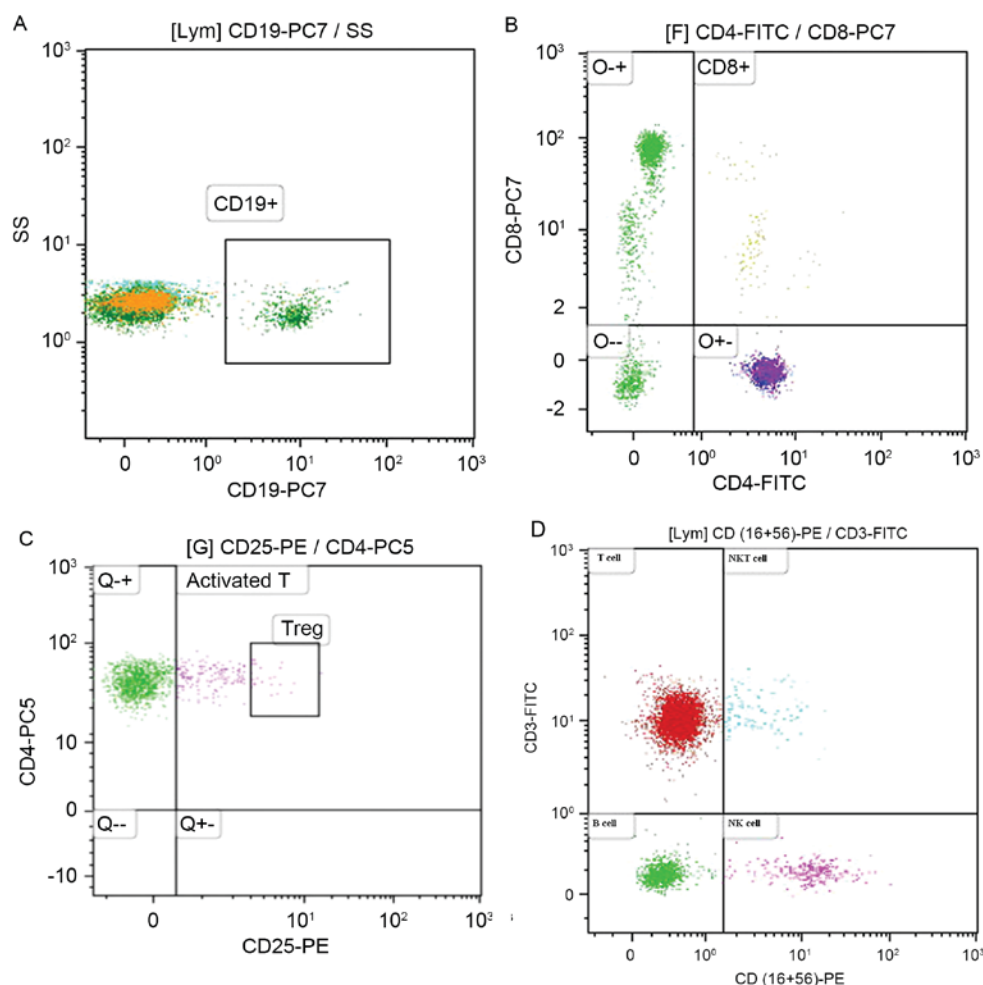


Figure 1. Distribution of lymphocyte subsets from peripheral blood as determined by flow cytometry. (A) Expression of CD19, and B lymphocyte is indicated as CD19<sup>+</sup>. (B) Co-expression of CD4 and CD8 by CD3<sup>+</sup> lymphocyte. A distinct subset of CD4<sup>+</sup>CD8<sup>-</sup> is shown in the lower left quadrant. (C) Co-expression of CD4 and CD25 by CD3<sup>+</sup> lymphocyte. The CD4<sup>+</sup>CD25<sup>+</sup> subset in the upper left quadrant is indicated as activated CD4<sup>+</sup> T lymphocyte, and a relatively distinct subset of CD4<sup>+</sup>CD25<sup>+</sup> expressing high CD25 is identified as Treg. (D) Co-expression of CD3 and CD16<sup>+</sup>56, A subset of CD3<sup>+</sup>CD16<sup>+</sup>CD56<sup>+</sup> in the upper right quadrant is indicated as NKT cells, A subset of CD3<sup>-</sup>CD16<sup>+</sup>CD56<sup>+</sup> in the lower right quadrant is indicated as NK cells. CD, cluster of differentiation; FITC, fluorescein isothiocyanate; lym, lymphocyte; NK cells, natural killer cells; NKT, natural killer T lymphocytes; PC, phycoerythrin cyanin; PE, phycoerythrin; Treg, regulatory T lymphocyte.



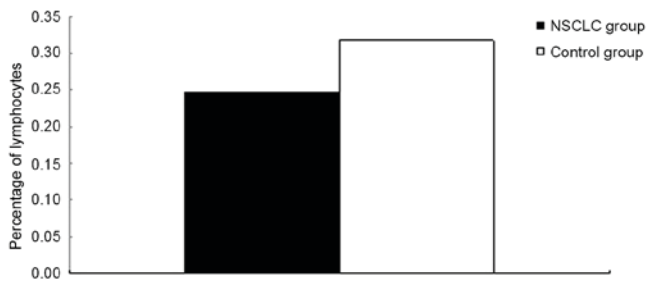


Figure 2. Comparison of lymphocytes in the NSCLC group and the control group. The percentage of lymphocytes is significantly lower in the NSCLC group compared with the control group.  $P=0.008$ . NSCLC, non-small cell lung cancer.

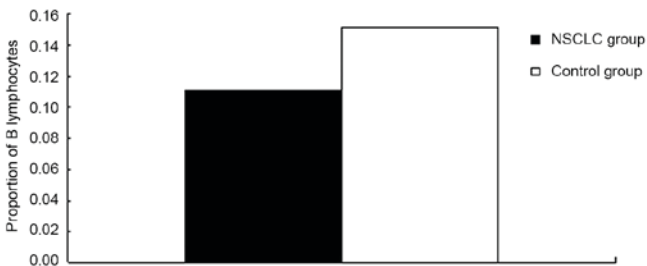


Figure 3. Comparison of B lymphocytes in the NSCLC group and the control group. The proportion of peripheral B lymphocytes is significantly lower in the NSCLC group compared with the control group.  $P<0.0001$ . NSCLC, non-small cell lung cancer.

been observed (1-3,27). As conventional therapeutic modalities are mostly concerned with prolonging the survival time of patients with marked toxic side effects, various types of immunotherapy have been attempted for decades (28). During the recent decade, the breakthrough finding of TME made tumor immunology another focus for investigation in tumor biology.

It is now clear that tumor cells have evolved to acquire various capacities to alter the topical milieu of tumor tissues, and to facilitate proliferation and invasion (29). The complicated mechanisms remain to be completely elucidated, and mechanisms that have been established includes exploitation of co-inhibitory checkpoint molecules through interaction between tumor cells and immune effector cells, recruitment of immune suppressive cells (Tregs and MDSCs), secretion of inhibitory cytokines and other agents, and reshaping the function of stromal and immune cells (6,8-10,30). These findings provide new strategy and targets for immunotherapy, and newly developed monoclonal agents based on these findings have achieved good clinical effects (13,14).

Since TME has a critical role in the biological behavior of tumor, it has been proposed that the involvement of the immune system is equally important in tumor development. Recently, evidence suggested that tumor cells were not only able to manipulate and reform local environment, but also exert systemic influence through tumor-derived cytokines and microvesicles. It was demonstrated that the systemic effects of the tumor could compromise distant tissues and organs so as to facilitate metastasis, and promote tumor growth (16). Nevertheless, there were also a number of studies (31) with inconsistent results (24). These seemingly contradictory

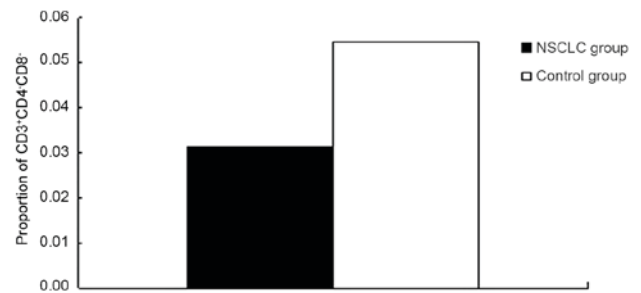


Figure 4. Comparison of CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> T cells in the NSCLC group and the control group. The proportion of peripheral CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> T cells is significantly lower in the NSCLC group compared with the control group.  $P=0.001$ . CD, cluster of differentiation; NSCLC, non-small cell lung cancer.

findings suggest the complexity of the systemic effects of tumors.

Theoretically since tumors are able to exert systemic effects, it is highly likely that they may adapt to the peripheral environment to facilitate progression and metastasis (32). Increasing evidence suggest that tumor is a systemic disease in that topical alteration within tumor tissue is closely associated with its systemic effect, for example the recruitment of Tregs into tumor site is accompanied by increased levels of Tregs in the peripheral blood (33,34).

In the present study, the changes in the proportions of peripheral lymphocytes and subpopulations were analyzed. The findings indicated that the percentage of lymphocytes in the NSCLC group was significantly lower compared with the control group ( $P=0.008$ ). This is in accordance with results in other types of malignant tumor (35,36).

To date, the reason or specific mechanisms for lymphopenia in malignant tumor is unclear. Ray-Coquard *et al* (37) proposed that a decreased lymphocyte count might reflect immunosuppressive condition in the tumor-bearing host, which suggest that the host tends to have an inadequate immunological reaction. A decreased lymphocyte count might also be a consequence of lympholysis caused by cytokines produced by tumor cells in the case of lymphoma (37).

The present study hypothesized that decreased lymphocyte count in tumor-bearing host is caused by tumor lesion, which is supported by evidence that elimination of tumor lesion by tumor antigen vaccination treatment is able to normalize decreased lymphocyte frequency (37). The results of the present study indicated that the percentage of lymphocytes in NSCLC with metastasis is significantly lower compared with the percentage in early stage NSCLC, which also support the hypothesis that decreased lymphocyte is associated with tumor progression. In addition, since tumor with metastasis is indicative of poor prognosis, the findings of the present study support that lymphopenia is an independent prognostic factor for overall and progression-free survival in cancer (37). However, the specific underlying mechanisms of how tumor affects the proportion of peripheral lymphocytes require further studies.

In the present study, it was observed that the proportion of CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> cells, a poorly known subpopulation in the peripheral blood, was significantly lower compared with the control group ( $P=0.001$ ), which has not been reported in any types of tumor previously. CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> lymphocytes

Table III. Comparison of lymphocyte subsets between subgroups I and II.

| Lymphocyte subsets                                     | Subgroup I  | Subgroup II | P-value <sup>a</sup> |
|--|-------------|-------------|----------------------|
| Lymphocyte   | 0.309±0.094 | 0.214±0.066 | <0.001               |
| CD3 <sup>+</sup>                                       | 0.716±0.106 | 0.689±0.084 | 0.354                |
| CD3 <sup>+</sup> CD4 <sup>+</sup>                      | 0.434±0.062 | 0.397±0.087 | 0.125                |
| CD3 <sup>+</sup> CD8 <sup>+</sup>                      | 0.254±0.088 | 0.275±0.065 | 0.196                |
| CD3 <sup>+</sup> CD4 <sup>+</sup> CD8 <sup>-</sup>     | 0.034±0.019 | 0.030±0.019 | 0.360                |
| CD3 <sup>+</sup> CD4 <sup>+</sup> CD25 <sup>+</sup>    | 0.086±0.025 | 0.098±0.025 | 0.875                |
| CD3 <sup>+</sup> CD4 <sup>+</sup> CD25 <sup>high</sup> | 0.002±0.000 | 0.002±0.004 | 0.883                |
| CD19 <sup>+</sup>                                      | 0.106±0.025 | 0.112±0.052 | 0.931                |
| CD3 <sup>+</sup> CD16 <sup>+</sup> CD56 <sup>+</sup>   | 0.063±0.048 | 0.090±0.061 | 0.070                |
| CD3 <sup>-</sup> CD16 <sup>+</sup> CD56 <sup>+</sup>   | 0.163±0.110 | 0.170±0.095 | 0.813                |
| CD4 <sup>+</sup> /CD8 <sup>+</sup>                     | 1.889±0.590 | 1.567±0.644 | 0.059                |

<sup>a</sup>Subgroup I vs. subgroup II. CD, cluster of differentiation.

Table IV. Comparison of the levels of cytokines between the NSCLC group and control group

| Cytokines     | NSCLC group (pg/ml) | Control group (pg/ml) | P-value <sup>a</sup> |
|---------------|---------------------|-----------------------|----------------------|
| IL-2          | 1.07±0.827          | 1.40±0.426            | 0.083                |
| IL-4          | 1.38±1.151          | 1.75±0.872            | 0.093                |
| IL-6          | 6.01±3.292          | 3.86±2.184            | 0.008                |
| IL-10         | 5.25±1.721          | 5.28±1.584            | 0.988                |
| IL-17A        | 2.01±1.226          | 1.99±0.435            | 0.081                |
| TNF- $\alpha$ | 0.35±0.379          | 0.35±0.301            | 0.652                |
| TGF- $\beta$  | 55,920±15,692       | 56,224±10,178         | 0.864                |
| IFN- $\gamma$ | 3.57±2.050          | 3.83±2.404            | 0.675                |

<sup>a</sup>NSCLC group vs. control group. IL, interleukin; TNF, tumor necrosis factor; TGF, transforming growth factor; IFN, interferon.



Figure 5. Comparison of lymphocytes in the NSCLC group and the control group. The percentage of lymphocytes in subgroup II (NSCLC with metastasis) is significantly lower compared with subgroup I (NSCLC without metastasis).  $P < 0.0001$ . NSCLC, non-small cell lung cancer.

are also known as DN T cell with  $\alpha\beta$ T-cell receptor (TCR) or  $\gamma\delta$ TCR. The CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> subpopulation is very small in

number and represents 1-3% of peripheral mononuclear cells. CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> cells are mainly distributed in the peripheral blood and lymph nodes (38,39). A previous study demonstrated that this novel subset of T cell might have a role in autoimmune disease, transplantation, viral infection and malignant tumor by exerting different functions (40). DN T cells are able to suppress CD4<sup>+</sup> and CD8<sup>+</sup> T cell-mediated response by eliminating effector T cells in murine models via the combination of Fas/Fas ligand or perforin/granzyme secretion, or suppressing the proliferation of activated T cells in humans via cell-cell interactions (41). Due to immunosuppressive properties of DN T cells, DN T cell has been proposed as a novel therapeutic target for autoimmune disease and transplantation. Studies have demonstrated that DN T cells are able to enhance the survival of organ allografts and xenografts (42). In human infections caused by the human immunodeficiency virus and Simian immunodeficiency virus, DN T cells are able to exert T helper cell-like functions in compensation for very low levels of CD4<sup>+</sup> T cells (43).

The roles of DN T cells in tumor have been gradually unraveled. Young *et al* (44) demonstrated that isolated DN T cells are able to kill lymphoma A20 cells *in vitro*, and prevent lymphoma cell growth in a mouse model (44). Merims *et al* (45) proposed a novel approach to expand DN T cells isolated from leukemia patients *in vitro*, and the results indicated that expanded DN T cells were able to kill leukemia blast cells isolated from patients *in vitro* via a perforin-dependent mechanism (45). Additionally, Voelkl *et al* (46) identified a DN T cell clone capable of killing melanoma cell isolated from a patient.

The findings of the present study suggest that tumor cells might decrease the proportion of peripheral DN T cells by an unidentified mechanism in order to create a favorable peripheral environment for distant organ metastasis since DN T cells are able to kill tumor cells directly in the absence of CD8<sup>+</sup> cells. If this finding is verified in future studies, DN T cells may be a promising therapeutic target for clinic prevention and control of metastasis. Further studies on the capability of DN T cells in the killing of NSCLC tumor cells would provide important insight.

Notably, the present study also observed that the proportion of peripheral B lymphocytes in the NSCLC group was significantly lower compared with the in control group ( $P<0.0001$ ), which has not previously been reported in any type of tumor. Except for its common function of antigen presentation and antibody production or secretion, the role of B lymphocytes in tumor has long been observed (47-49). Many studies using murine models demonstrated that B cells were able to markedly suppress antitumor immunity in various types of tumor. In the B cell deficient mice (BCDM) model, slow growth or regression of implanted tumors was associated with indicators of antitumor immune responses, including dense infiltration of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the tumor bed, increased Th1 response and enhanced Cytotoxic T lymphocyte-mediated cytotoxicity against tumor cells (48). By contrast, tumor growth restored when B cells were transplanted into BCDM or wild-type mice (48-50).

A subset of B cells was recognized with immunosuppressive function, namely B regulatory cells (Breg) (51). Breg has been identified with different phenotypes in different settings. Studies indicated that B regs were able to induce primary CD4<sup>+</sup> T cell differentiation into the Th1/Th2 type (52). However, the mechanism and the specific conditions that enable B reg cells to exert this function are unclear. Moreover, B regs have been demonstrated to be able to promote the conversion of naive CD4<sup>+</sup> T cells into Tregs, Tregs have been established to exert an important immunosuppressive role in TME. A number of studies support that the observation that the effect of Bregs in tumor may be mediated by the conversion of Tregs (53). In tumor models, Bregs have been observed to infiltrate tumor tissues. Tumor infiltrating Bregs [(TIL, tumor-infiltrating lymphocytes)-Bregs] are able to express various immunosuppressive molecules, which may mediate their immunosuppressive effect (53). However, the role of Bregs in tumor remains controversial since studies indicate that TIL-Breg is associated with improved survival (54), and some studies indicated that B cells may have a protective against tumor (55). Based on these studies, it is hypothesized that the preliminary findings of the present study indicate that B cells may be recruited into tumor tissues.

Circulating cytokine is closely associated with systemic and local immune status in disease, such as cancer. In the present study, it was observed that the level of IL-6 in the NSCLC group was significantly higher compared with the control group ( $P=0.008$ ), whereas the proportions of the other 7 cytokines, including IFN- $\gamma$ , TNF- $\alpha$ , TGF- $\beta$ , IL-2, IL-4, IL-10 and IL-17A, were not significantly different between the NSCLC group and the control group. In addition, the levels of none of the cytokines was significantly different between subgroups I and II. Previous *in vitro* experiments demonstrated that IL-6 may have a dual role in antitumor immunity. IL-6 is able to promote tumor growth through downstream mediators and help sustain immunosuppressive milieu in TME (56). Additionally, IL-6 is also an important mediator of T cell recruitment to lymph nodes and tumor site, and skewing the conversion of CD4<sup>+</sup> T cells from Tregs to a Th17 phenotype (56). Lippitz (57) also concluded that circulating IL-6 level is elevated in cancer patients and is also correlated with poor prognosis (50). Moreover, Lippitz (57)

proposed that systemic cytokine cascade is characteristic of cancer (50).

The authors of the present study support the hypothesis that systemic cytokine changes are closely associated with tumor progression, which may be regulated by the tumor considering tumor cells are able to secrete various pro-tumor cytokines. Although, in the present study, significant changes in the levels of cytokines were not observed, which is inconsistent with the analysis of Lippitz (57), the reason may be due to a relatively smaller sample size used in the present study. The present authors support the hypothesis that systemic cytokine cascade exists in patients with tumors, which reflects tumor stage and host immune status. Furthermore, these changes in the level of cytokines may be potential targets for immunotherapy.

In conclusion, it was observed in the present study that in NSCLC patients, the proportion of lymphocytes and two subpopulations (CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> and CD19<sup>+</sup>) were significantly different between NSCLC patients and healthy controls.

The level of circulating IL-6 in NSCLC patients was also significantly higher in the NSCLC group compared with healthy controls. These preliminary results support the hypothesis that peripheral immune system is adapted by tumor lesion, and a further question is whether it is a strategy adopted by tumor cells in order to facilitate progression and metastasis. Further studies which focus on the role of peripheral B cells and DN T cells in tumor may determine if these cells have a role in immunosurveillance and provide a novel strategy for immunotherapy.

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#### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

HL analyzed the data and was a major contributor in writing the manuscript. XC made substantial contribution to the conception and design of study and gave the final approval of the version to be published. JZ had major role in designing the study. GX and YS made substantial contribution in analysis and interpretation of the data. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

This study was approved by Research Ethics Committee of the General Hospital of People's Liberation Army (approval no. S2015-02-11). All patients provided informed consent to participate this study.

## Consent for publication

Informed consent was obtained from all patients included in this study for publication of the associated data and the accompanying image.

## Competing interests

The authors declare that they have no competing interests.

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