The Importance of Alterations in Innate Lymphoid Cell Subsets in Patients with Non-Small Cell Lung Cancer and their Role in Tumorigenesis

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Abstract

Aim: Non-small cell lung cancer (NSCLC) is one of the leading causes of cancer-related morbidity and mortality. Diverse functions of innate lymphoid cells (ILCs) and NK cell subsets are investigated thoroughly in cancer immunotherapy. ILC and recently described NK cell subsets in NSCLC patients' blood samples and tumor draining lymph nodes were investigated.

Methods: The study included chemotherapy and/or radiotherapy-naive NSCLC patients with clinical stage T1-4N0-2M0 who underwent video-assisted mediastinal lymphadenectomy and 14 healthy controls. Mononuclear cells were isolated from peripheral blood of both groups and mediastinal lymph nodes of NSCLC patients. NK cells and ILC subsets were analyzed by flow cytometry.

Results: Total NK cells are shown to be increased in peripheral blood of NSCLC patients compared to lymph nodes while the ratio of $CD56^{dim}CD16^{-}$ exhausted NK cells is higher in lymph nodes than in blood samples of NSCLC patients. Compared to control group, peripheral blood ILC1 cells were lower in NSCLC patients, however ILC2 and ILC3 cells were significantly increased. However, mediastinal lymph nodes of NSCLC patients had decreased ratio of ILC2 and increased ratio of ILC3 cells than in peripheral blood of patients. NSCLC patients had significantly increased ratio of $NKp44^{-}$ILC3 cells and decreased ratio of $NKp44^{+}$ILC3 in lymph nodes.

Conclusion: Decreased ratio of ILC1 cells is an important indicator of impaired anti-tumoral response. Increased in the ratio of $NKp44^{-}$ILC3 cells in NSCLC patients may potentially contribute to tumor progression. These findings highlight the distinct roles of ILCs, which play a pivotal role in the pathogenesis of lung cancer.

Keywords: *NSCLC, NK cells, ILC1, ILC2, ILC3, immunotherapy*

1. Introduction

The goal of cancer treatments is to improve survival rates, and recent advancements in immunotherapies and our understanding of tumor immunity have been considered in a new era in cancer - treatment. However, lung cancer has still high mortality rates[1-2].

Histologically, lung cancer can be divided into two main groups: Non-Small Cell Lung Cancer (NSCLC), which accounts for approximately 85% of all cases, and Small Cell Lung Cancer (SCLC), which represents about 15% of all lung cancers[3]. Nonmetastatic (stage I-IIIA) NSCLC is generally treated with multimodality treatment approaches including surgery, radiotherapy, chemotherapy, and immunotherapy[4-5]. Unfortunately, the majority of lung cancer cases are diagnosed at late stages (stage IIIB-IV) with distant metastasis and/or local invasion. As a result, there is currently significant research focus on enhancing antitumoral immunity in the field of oncology[6].

Recent studies have revealed the significance of innate immunity, and its contribution to antitumor immunity. A distinct subset of lymphocytes known as innate lymphoid cells (ILCs) has gained attention in this context. ILCs encompass a diverse group of cells, in-

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cluding cytokine-producing ILCs and natural killer (NK) cells. They are recently classified into three different subgroups according to their cytokine secretion and transcription factor expression: group 1 ILC (comprising ILC1s and NK cells), ILC2 and ILC3. Lymphoid tissue inducer cells (LTi) are a distinct group of ILCs but thought to belong to group-3 ILCs[7]. There is homology between T helper cell subsets and ILCs. Group 1 ILCs is a counterpart of Th1 consist of NK and ILC1 cells. These cells secrete tumor necrosis factor (TNF)-α and interferon (IFN)-γ, playing a key role in antitumor immunity. However, unlike ILC1s and other ILCs, NK cells possess cytotoxic functions. While NK cells can circulate in the bloodstream, ILC1 cells predominantly reside in various tissues[7-8]. NK cells employ several mechanisms in their antitumor activity, including antigen-dependent cell cytotoxicity, induction of target cell apoptosis via FASL and TRAIL signaling, and target cell lysis using cytolytic granules containing molecules such as perforin and granzyme[9]. The overall effect of NK cells is the enhancement of both innate and adaptive immunity, leading to a robust antitumor response.

ILC2 cell subset, similar to Th2 cells, secrete cytokines such as IL-4, IL-5 and IL-13 which contribute to type 2 immune responses. ILC2 cells originate from the progenitors in bone marrow and are found in various organs such as lung, intestines, adipose tissue, and the skin[11]. It has been shown that an increased ratio of ILC2s in gastric cancer patients creates an immunosuppressive microenvironment that facilitates tumorigenesis in this group[12]. ILC3 cells functionally resemble Th17 cells and secrete IL-17 and IL-22. A unique subgroup of ILC3s, lymphoid tissue inducer cells (LTi) that play a role in modulating lymphoid tissues during inflammatory conditions and even in embryological development[7]. Additionally, ILC3 cells expressing the Natural Cytotoxicity Receptor (NCR) have been found to enhance antitumoral immunity by promoting the formation of ectopic lymphoid structures in NSCLC patients. These findings are associated with a better prognosis in terms of clinical outcomes for lung cancer patients[13].

ILCs play critical regulatory roles in various inflammatory conditions including autoimmune diseases, infections, and cancer. They exhibit distinct functions depending on the specific tissue and context in which they are found[14]. Recent research has shown that ILCs possess plasticity, meaning they can convert from one subgroup to another, further complicating the categorization of their roles[15]. This study focuses on the identification of distinct subgroups of innate lymphoid cells (ILCs) and examines their distribution in the peripheral blood of both non-small cell lung cancer (NSCLC) patients and healthy controls. Additionally, the presence of ILC subgroups in the tumor draining lymph nodes of NSCLC patients was investigated. The primary objective of this study was to gain insight into the potential impact of alterations in ILC and natural killer (NK) cell subsets on tumorigenesis in NSCLC patients. In addition to the primary objective, this study also aimed to explore and compare the differences between the distribution of ILC subgroups in the peripheral blood and tumor draining lymph nodes of NSCLC patients. By examining these distinct anatomical sites, the secondary outcome was to provide a comprehensive understanding of the potential variances in ILC composition within the same individuals affected by NSCLC.

2. Materials and methods

2.1. Study Population

Thirteen patients diagnosed with operable non-small cell lung cancer (NSCLC) and scheduled to undergo Video-Assisted Mediastinal Lymphadenectomy (VAMLA) between January 2018 and December 2019 were prospectively enrolled in the study. Prior to surgery, all patients underwent a comprehensive preoperative staging evaluation, including transthoracic or bronchoscopy-assisted biopsy, computed tomography, positron emission computed tomography, and cranial magnetic resonance imaging. Staging of the patients was performed according to the 8th edition of the TNM staging system for lung cancer, and they were classified as having a disease stage of cT1-4N0-3M0-1A. None of the patients had a history of chemotherapy or radiotherapy. Bilateral mediastinal lymph node dissection was performed using the VAMLA technique for all patients. Fourteen age- and sex-matched healthy individuals with no history of malignancy or self-reported chronic diseases were included as the control group (Table 1), and peripheral blood samples were collected from these individuals. The study protocol was approved by the institutional review board (document 2017/262587) and adhered to the principles outlined in the Declaration of Helsinki. Written informed consent was obtained from all participants.

2.2. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples using density gradient centrifugation with Ficoll-Paque (Histopaque-1077; Biochrom, UK). Heparin-anticoagulated blood was centrifuged at 800 × g for 20 minutes to remove plasma. The remaining blood was diluted with an equal volume of phosphate-buffered saline solution (PBS) (Biochrom, UK). The cells were then adjusted to a final concentration of 1 × 106 cells/mL in complete RPMI-1640 medium, which consisted of RPMI supplemented with 10% fetal calf serum, 1% L-glutamine, and 1% anti-mycotic and antibiotic solutions (all from Sigma Chem. Co., Germany).

2.3. Isolation of lymphocytes from mediastinal lymph nodes

The lymph node samples after VAMLA procedure were transferred to pathologist to assess the presence of any tumor involvement or other pathologic conditions. Remaining lymph nodes that were not needed for pathological evaluation were transferred to the laboratory in complete RPMI-1640 medium. The lymph nodes were transferred to a sterile petri dish, crushed into smaller pieces using a sterile scalpel on ice, and the crushed lymph node samples were filtered through a 70-µm cell strainer (BD Biosciences, USA). To isolate mononuclear cells, cell suspension was centrifuged by the Ficoll-Hypaque gradient and centrifuged at 800xg for 20 minutes.[16] After centrifugation, lymphocyte-rich mononuclear layer was collected. The washing process was repeated two times then the cells were re-suspended in a complete RPMI-1640 culture medium.

2.4. Determination of NK cell subsets from peripheral blood and lymph nodes

Freshly isolated mononuclear cells from peripheral blood and mediastinal lymph nodes were washed and re-suspended in staining buffer (PBS containing 2% fetal calf serum) and surface staining was done using fluorochrome-conjugated anti-human CD3-FITC, anti-human CD16-Alexa Fluor 700 and anti-human CD56-PerCp (all from Biolegend, USA) monoclonal antibodies. Specific subpopulations were identified by comparison to autofluorescent controls. Tubes were incubated for 30 min at room temperature and were washed with staining buffer. After washing twice with staining buffer, cells were re-suspended in 500 µL PBS with 1% paraformaldehyde and acquired on FACSAria II (Becton Dickinson, San Jose, CA, USA). This instrument allows for the analysis of individual cells based on their fluorescence properties. The acquired data were analyzed using FACS DIVA (BD Biosciences, USA) software. FlowJo software (BD Biosciences, USA) was used for further analysis of the flow cytometric data. NK cell subsets were further divided based on the expression levels of CD16 and CD56 into six subsets:

$CD56^{bright}CD16^{-}$, $CD56b^{bright}CD16^{dim}$, $CD56^{dim}CD16^{-}$, $CD56^{dim}CD16^{dim}$, $CD56^{dim}CD16^{bright}$ and $CD56^{-}CD16^{bright}$ cells[17].

2.5. Flow cytometric determination of ILC subsets from peripheral blood and lymph nodes

PBMCs were isolated from heparinized blood and lymph node

samples and the cells were labeled a panel of conventional FITC labeled lineage negative markers (anti-human CD1a, anti-human CD4, anti-human TCRαβ, anti-human TCRγδ, anti-human CD3, anti-human CD11c, anti-human CD14, anti-human CD94, anti-human CD19, anti-human CD123, anti-human CD303, anti-human CD34, anti-human FcεR1, anti-human CD16), Viability Dye-APC/CY7, anti-human NKp44-PE, anti-human CD161-PerCp.Cy5.5, anti-human CD127-PE/Cy7, anti-human CRTH2 (CD294)-APC, anti-human c-kit (CD117)-BV421 and anti-human CD45-BV510 monoclonal antibodies (All from Biolegend, San Diego, CA, USA) and incubated for 30 min at room temperature in the dark. After incubation, cells were washed by PBS and fixed in PBS with 1% paraformaldehyde. The auto-fluorescent tube was used as an isotypic control for analysis. Data were acquired on a FACSAria II by using the FACSDiva operating system software (BD Biosciences, San Jose, CA, USA). Flow cytometric analyses were performed using FlowJo software (BD Biosciences, USA).

2.6. Statistical Analysis

The data analysis was performed using IBM SPSS (Statistical Package for Social Sciences) for Mac 21.0 software. The normality of the data distribution was assessed using the Kolmogorov-Smirnov test. The comparison of independent variables was conducted using the Mann-Whitney U test. Statistical significance was determined at a p-value < 0.05. Graphical representations were generated using GraphPad Prism 9.0 software (GraphPad Software Inc., La Jolla, USA).

3. Results

3.1. NK cells with exhausted phenotype increased in mediastinal lymph nodes of NSCLC patients

The ratio of total NK cells was significantly lower in the tumor draining mediastinal lymph nodes of the patients compared to their blood levels (p<0.001). The ratio of $CD56^{bright}CD16^{-}$ cytokine-secreting NK, $CD56^{bright}CD16^{dim}$ NK, $CD56^{dim}CD16^{bright}$ mature cytotoxic NK and $CD56^{-}CD16^{bright}$ NK cells were significantly reduced in the tumor draining mediastinal lymph nodes of the patients compared to their peripheral blood levels (p=0.001, p=0.001, p<0.001 and p=0.005, respectively). $CD56^{dim}CD16^{-}$ exhausted NK cells increased in the tumor draining mediastinal lymph nodes compared to peripheral blood of patients and healthy controls (p<0.001 and p<0.001, respectively). $CD56^{-}CD16^{bright}$ NK cells were increased (p=0.003), while $CD56^{dim}CD16^{bright}$ NK cells were decreased in the peripheral blood of the patients compared to healthy controls (p=0.008) (Figure 1).

3.2. Diminished ILC1 but increased ILC2 and ILC3 cells in active NSCLC patients

The ratio of the total ILCs did not change between the peripheral blood of the patients and their mediastinal lymph nodes or control blood. However, the distribution of the ILC subsets varied significantly in patients and controls. The ratio of $CD45^{+}Lineage^{-}CD127^{+}CD161^{+}CRTH2^{-}c\text{-}kit^{-}$ ILC1 cells was significantly reduced in the peripheral blood and mediastinal lymph nodes of the patients compared to the controls (p=0.018 and p=0.014, respectively). This is functionally relevant, as ILC1 cells represent IFN-g secreting population of ILCs and have important antitumoral functions. Interestingly, the ratio of $CD45^{+}Lineage^{-}CD127^{+}CD161^{+}CRTH2^{+}c\text{-}kit^{+}$ ILC2 cells was significantly higher in the peripheral blood of the patients compared to healthy subjects, while it was significantly lower in the mediastinal lymph nodes of the patients. The expression of $CD45^{+}$ $Lineage^{-}CD127^{+}CD161^{+}CRTH2^{-}c\text{-}kit^{+}$ ILC3 cells increased significantly in the peripheral blood of the patients compared to the healthy individuals (p=0.006) and was further increased in the mediastinal lymph nodes of the patients compared to their blood levels (p=0.022). ILC3 cells have also two distinct subtypes according to their cytokine patterns. NCR negative ILC3 cells increased in the peripheral blood and mediastinal lymph nodes of the patients compared to controls (p=0.035 and p=0.030, respectively). The ratio of IL-22 secreting NCR positive $NKp44^{+}$ILC3 cells decreased in the peripheral blood and mediastinal lymph nodes of the patients compared to control blood (p=0.022 and p=0.017, respectively) (Figure 2A and 2B).

Table 1

Demographic data of study and control groups

	Patients (n=13)	Controls (n=14)
Gender		
• Female, n (%)	2 (15)	4 (28.6)
• Male, n (%)	11 (85)	10 (71.4)
Age (years, mean ± SD)	64.2 ± 6.1	54.3 ± 8.7
Diagnosis		
• Adenocarcinoma	4 (30.7%)	-
• Squamous cell carcinoma	6 (46.2 %)	-
• NSCLC, not otherwise classified	3 (23.1%)	-
Clinical Stage		
• Stage I	4 (30.7%)	-
• Stage II	1 (7.7%)	-
• Stage III	5 (38.5%)	-
• Stage IV	3 (23.1%)	-
Surgery		
Lobectomy	4 (66.6%)	
Bilobectomy	1 (16.6%)	
Pneumonectomy	1 (16.6%)	

SD; standard deviation, NSCLC; non-small cell lung cancer

4. Discussion

Immunotherapies are widely used in the treatment of various cancers, including lung cancer. These treatments primarily involve targeting inhibition of immune checkpoint receptors on T lymphocytes to enhance the immune response against cancer cells[18]. However, despite significant treatments, effectively treating cancer remains a challenging task. In recent years, the importance of innate immunity has been well demonstrated, shedding light on the importance on ILC and NK cells[19]. This study investigated the ratio of NK and ILC subsets within peripheral blood and tumor draining lymph nodes of NSCLC patients. The findings revealed remarkable changes in the expression of both NK cells and ILC subsets in these patients. NK cells, unlike T lymphocytes can recognize target cells without the need for interaction with MHC molecules[20]. Studies have shown that increased number of NK cells within the tumor microenvironment (TME) in solid tumors, including lung cancer and colon cancer, is associated with better prognosis, regardless of the ongoing cancer treatments[21-23].

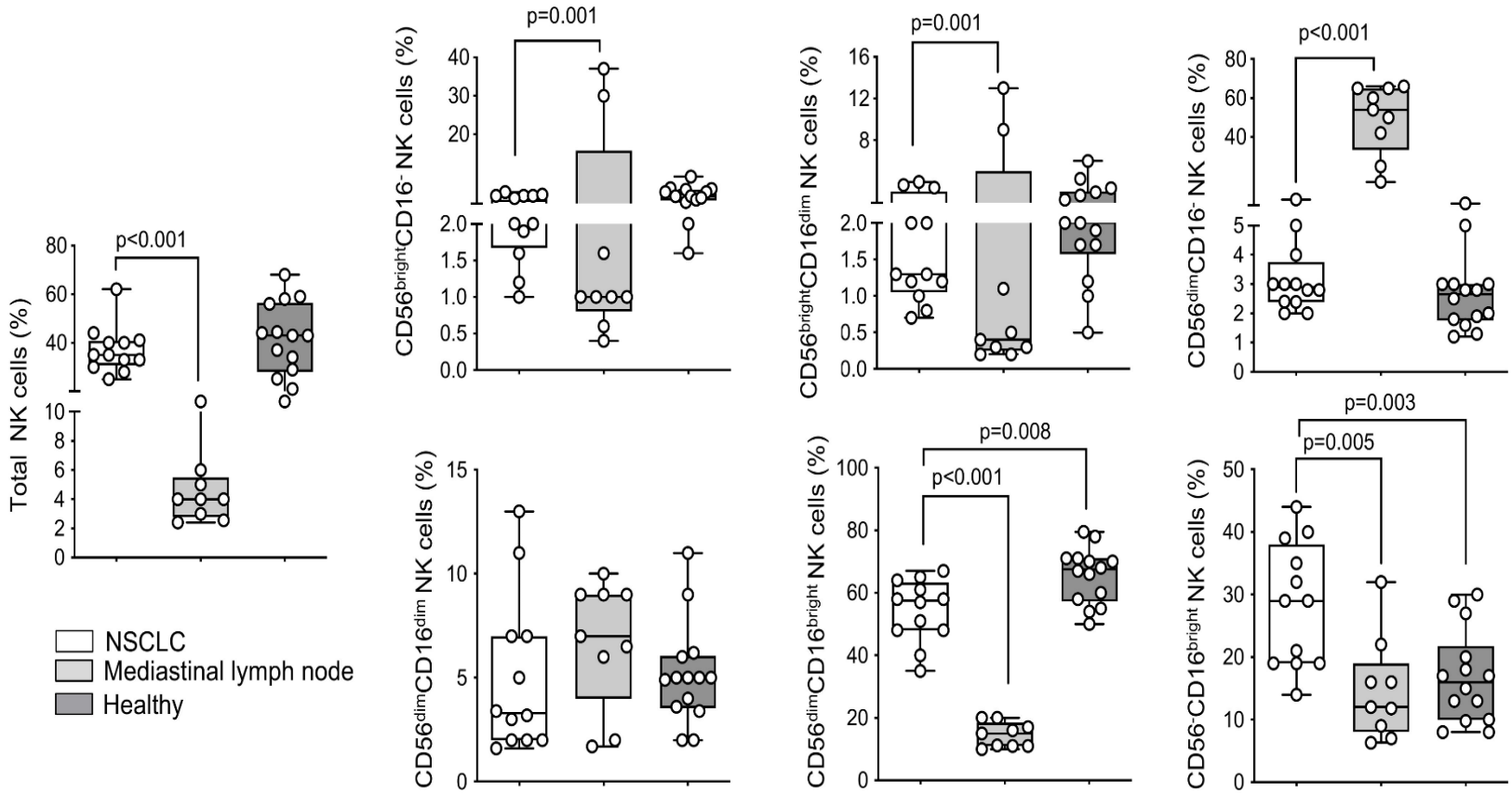

Figure 1

Percentages of six different NK cell subsets in peripheral blood and lymph node samples in NSCLC and healthy subjects. NK cells were classified into six subsets as follows: $CD56^{bright}CD16^{-}$, $CD56^{dim}CD16^{dim}$, $CD56^{bright}CD16^{dim}$, $CD56^{dim}CD16^{bright}$, $CD56^{dim}CD16^{-}$ and $CD56^{-}CD16^{bright}$ NK cells. Percentages of the six different NK subsets and total NK cells in peripheral blood and mediastinal lymph nodes of NSCLC patients and peripheral blood of controls were shown as bar graphs. Differences were considered significant when $p < 0.05$.

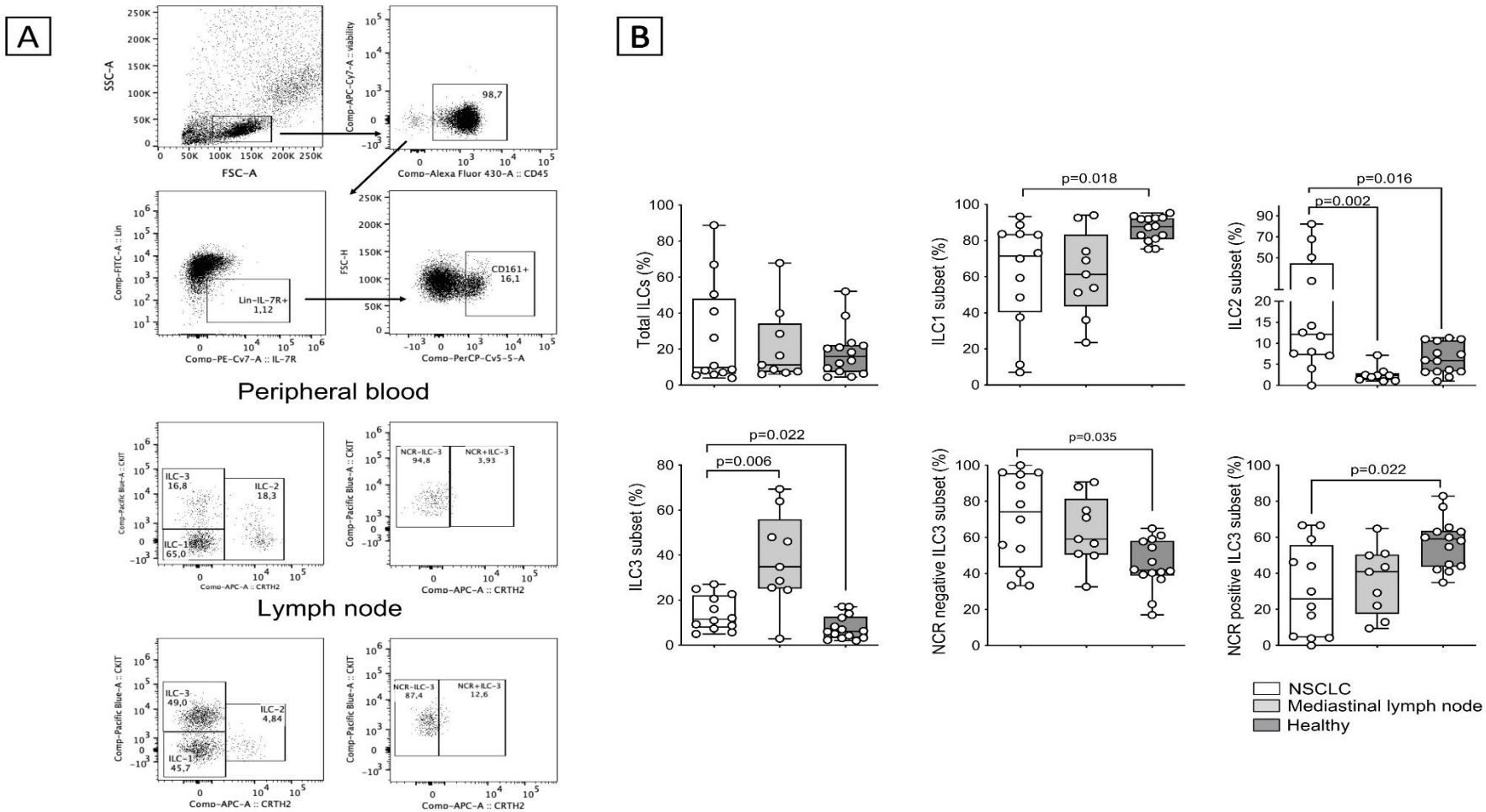

Figure 2

Flow cytometric gating strategy for identifying peripheral blood and lymph node ILC subsets. CD45+ cells were gated by excluding dead cells within the lymphocyte gate. ILCs were defined as Lineage-CD127+ cells. The CD161+ cells were selected as the total ILC gate. ILCs were further classified into ILC1, ILC2, and ILC3 subsets based on the expression of c-kit and/or CRTH2. Within the ILC3 subset, cells were further divided into NKp44-ILC3 and NKp44+ILC3 subsets based on NKp44 expression. (A) The percentages of total ILC, ILC1, ILC2, ILC3, and ILC3 subsets (NKp44+ILC3 and NKp44-ILC3) were analyzed in peripheral blood and lymph nodes of NSCLC patients and healthy subjects. (B) The median values are presented as bars. Statistical analysis was performed using the Mann Whitney U-test, and differences were considered significant when $p < 0.05$.

It is not only the number of NK cells present in the TME that influences antitumor immunity, but also the functionality of these NK cells. A population study conducted in Japan showed that patients with decreased NK cell cytotoxic activity have a higher probability of developing cancer.[24] Consistent with these findings, the current study showed a decreased ratio of cytotoxic $CD56^{dim}CD16^{bright}$ NK cells in the peripheral blood of NSCLC patients compared to healthy controls. Furthermore, we found a significant increase in exhausted $CD56^{dim}CD16^{-}$exhausted NK cells and a significant reduction in mature cytotoxic $CD56^{dim}CD16^{bright}$ NK cells in the mediastinal tumor draining lymph nodes of NSCLC patients. These findings suggest that cytotoxic and cytokine-secreting NK cells play crucial roles in antitumor immunity and are significantly diminished in NSCLC patients. The dominant NK cell subset observed in NSCLC patients is the exhausted NK cell phenotype. Overcoming NK cell exhaustion may represent a potential future treatment approach for NSCLC patients.

ILC1 cells play a vital role in promoting antitumor immunity by reducing the risk of progressive disease and the development of distant metastasis through their secretion of IFN-γ.[25] Verma et al[26], showed that ILC1 cells secreting IFN-γ are crucial for maintaining antitumor immunity, while ILC1 cells that produce low levels of IFN-γ possess tumor-promoting capabilities and are associated with a worse prognosis in patients with NSCLC. In our own investigation, we observed a significant decrease in the ratio of ILC1 cells in the peripheral blood and tumor draining mediastinal lymph nodes of NSCLC patients. These findings indicate that lower levels of ILC1 cells are linked to impaired antitumor activity.

ILC2 cells play important roles in type 2 immune responses, but their exact roles in anti-tumoral immunity are complicated and not fully understood. Recent studies have produced conflicting results, suggesting that ILC2 cells can have both anti-tumoral and tumor-promoting effects. Lung cancer, patients with a higher ratio of ILC2 cells have shown improved antitumor immunity and a decreased risk for distant metastasis.[27] However, in bladder cancer patients, pro-tumorigenic cytokines such as IL-4 and IL-13 secreted by ILC2 cells have been found to promote an immunosuppressive TME. This leads to a higher risk of recurrence and a poor prognosis.[28] In our current study, we demonstrated that a significantly higher ratio of ILC2 cells in the peripheral blood of NSCLC patients compared to healthy controls. This suggests that ILC2 cells may have tumor-promoting effects in NSCLC. Similar to our findings, Zhang et al. demonstrated in their study that the ratio of ILC2 cells in the peripheral blood and tumor tissue of NSCLC patients was higher compared to healthy controls.[29] Intriguingly, our study revealed that the ratio of ILC2 cells in tumor-draining lymph nodes was lower compared to the peripheral blood of NSCLC patients. Our findings suggest that higher ILC2 cells in peripheral blood of patients may have tumor promoting effects.

ILC3 cells comprise a significant subgroup of including NCR^{+}ILC3, NCR^{-}ILC3 and LTi cells. These cells have been found to exhibit dual effects, with both tumor-promoting and antitumor functions in different cancer types.[30] $NKp44^{-}$ILC-3 cells, which are a subset of NCR^{-}ILC-3 cells, are known to predominantly secrete IL-17. Studies have shown that IL-17-secreting NCR^{-}ILC3 cells can play a role in promoting tumor growth, particularly in a mouse model of hepatocellular carcinoma.[31] In human colorectal cancers, it has been observed that NCR^{-}ILC3 cells have tumorigenic functions and are associated with worse clinical outcomes.[32] Consistent with these previous observations, our study demonstrated that the ratio of NCR^{-}ILC-3 cells were higher in the peripheral blood and mediastinal lymph nodes of the patients compared to the controls. These findings suggest a potential role for NCR^{-}ILC3 cells in promoting tumor growth and progression in NSCLC. Recent evidence has revealed the crucial role of $NKp44^{+}$ILC-3 cells (NCR^{+}ILC-3 cells) in secreting IL-22 while exhibiting minimal expression of IL-17. It has been observed that tertiary lymphoid structures, primarily formed by NCR^{+}ILC-3 cells, and an increased infiltration of higher NCR^{+}ILC-3 cells within these structures are associated with early-stage tumors and improved prognosis.[13] Similarly, in this study we demonstrated that the ratio of NCR positive $NKp44^{+}$ILC-3 cells were lower in both peripheral blood and mediastinal lymph nodes of the patients compared to healthy controls.

One limitation of this study is the ethical consideration that prevents the excision of healthy mediastinal lymph nodes for direct comparison with the lymph nodes of NSCLC patients. Due to ethical constraints, it was not feasible to obtain healthy lymph nodes for analysis. Consequently, the study was unable to directly investigate the differences in ILC distribution between healthy and tumor draining lymph nodes, which could have provided valuable comparative insights.

On the other hand, a significant strength of this study lies in its comprehensive assessment of ILC distribution. By examining not only the peripheral blood but also the lymph nodes of NSCLC patients, the study offers a more holistic understanding of ILC involvement in the disease. Furthermore, the study benefits from incorporating the most current classification of NK cells, facilitating a clearer demonstration of the increased exhausted pattern observed in NSCLC patients. This comprehensive approach enhances the significance and relevance of the study's findings.

5. Conclusions

Our study demonstrated an increased exhausted phenotype of ILCs and a decreased ratio of effector ILCs in NSCLC. We observed a decreased ratio of ILC1 cells and $NKp44^{+}$ILC-3 cells, along with an increased ratio of $NKp44^{-}$ILC-3 cells, which suggests tumorigenic changes in the innate immune response of NSCLC. Similar to changes in ILCs, NK cells showed a significant functional change from cytotoxic phenotype to exhausted phenotype. These alterations may contribute to a shift in the TME from an antitumor state to a tumorigenic state for innate immune cells. However, further studies are needed to explore strategies for revitalizing ILCs and NK cell function and enhancing their antitumor capabilities in the context of NSCLC.

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None.

Statement of ethics

This study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved by for this study the Cukurova University Institution Ethics Committee (2010-Thesis number 247902).

Conflict of interest statement

The authors declare that they have no financial conflict of interest with regard to the content of this report.

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Author contributions

Collection of the data, draft: MU, SS, Writing of the article,

performed the analysis, review of the literature: SY, EA, Critical review of the article, design of the study: HB,UT,EK

All authors read and approved the final manuscript.

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