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ORIGINAL RESEARCH

Impact of Chemotherapy on Circulating Lymphocyte Subsets in Lung Cancer Patients

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Purpose: Lung cancer remains a leading cause of cancer-related death and chemotherapy stands as a fundamental component in therapy. Chemotherapy-induced myelosuppression encompasses a spectrum of hematological declines, including not only neutrophils but also lymphocytes, hemoglobin levels and platelets. This retrospective cohort study investigates alterations in peripheral blood lymphocyte subsets. By uncovering these changes, our goal is to refine patient management strategies, ensuring that the benefits of chemotherapy are maximized while minimizing its detrimental effects.

Patients and Methods: We retrospectively analyzed 159 lung cancer patients. Patients were categorized as "NT" (n=108, no previous anti-tumor therapy), and "PT" (n=51, prior therapy followed by at least a two-month treatment-free interval). Post-chemotherapy, patients were reassessed and grouped into "EarlyCycle" for those who underwent four or fewer cycles, and "LateCycle" for those who underwent more than four cycles.

Results: The study focused on analyzing the percentages of lymphocyte subsets, including T cells (CD4+, CD8+), B cells, and natural killer (NK) cells, across these groups. For T cells, the EarlyCycle group exhibited a significant increase compared to NT (0.7783 vs 0.7271; p=0.0017) and PT (0.7783 vs 0.6804; p=1.6e-05). B cells showed a significant decrease from NT to LateCycle (0.1014 vs 0.0817; p=2.2e-05) and from PT to LateCycle (0.1317 vs 0.0817; p=6.2e-10). NK cells significantly decreased in the EarlyCycle group compared to NT (0.1109 vs 0.1462; p=0.00816) and PT (0.1109 vs 0.1513; p=0.00992), with no significant change in the LateCycle group compared to either NT or PT (p>0.05).

Conclusion: Chemotherapy significantly affects lymphocyte subsets in a treatment-specific manner. The EarlyCycle group experienced a reduction in NK cell and an increase in T cell, suggesting a damage of innate immunity and an early shift towards adaptive immunity. The LateCycle group showed a substantial decrease in B cell, indicating a delayed effect on humoral immunity components.

Keywords: chemotherapy, lung cancer, myelosuppression, peripheral blood, lymphocyte subsets

Introduction

Lung cancer remains a leading cause of cancer-related mortality worldwide.[1,2] Among the various treatment modalities, chemotherapy is fundamental but is often accompanied by myelosuppression.[3,4] This condition, characterized by the suppression of bone marrow activity, leads to a drop in blood cell counts not only neutrophils but also lymphocytes, increasing the risk of infection and potentially interrupting treatment continuity.[3,5] While the effect of myelosuppression on neutrophils is well-documented and mitigated by agents like granulocyte-colony stimulating factor (G-CSF),[6] its impact on lymphocytes—crucial components of the immune system—is not as well understood.[7,8] Lymphocytes, including T cells, B cells, and NK cells, are essential for maintaining immune surveillance and balance.[9] And at the same time, it is a valuable predictor of prognosis in lung cancer.[10] Each subset plays a unique role in the body's defense mechanisms: T cells are involved in cellular immunity,[11,12] B cells in humoral responses,[13] and NK cells in innate immunity.[14] While granulocyte-colony stimulating factor (G-CSF) effectively counters neutropenia, it does not alleviate the broader lymphocyte suppression that could leave patients susceptible to infections and potentially compromise the efficacy of anti-cancer therapies. Some studies have shown that chemotherapy can lead to lymphopenia.[15–17] However,

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the impact varies depending on the type and regimen of chemotherapy used.[17,18] Some chemotherapeutic agents have been observed to selectively deplete certain lymphocyte subsets, while others may induce immunogenic cell death, potentially enhancing anti-tumor immunity.[19]

The primary objective is to investigate the variations in blood lymphocyte subsets in lung cancer patients undergoing chemotherapy. Specifically, we aim to: Quantify the percentages of lymphocyte subsets—T cells (including CD4+ and CD8+), B cells, and NK cells—before and after chemotherapy in patients without prior anti-tumor therapy (NT group) and those with previous treatments (PT group). There are different T cells (CD45+CD3+) subsets exerting distinct immunoregulatory effects: helper T Cells (CD45+CD3+CD4+), cytotoxic T Cells (CD45+CD3+CD8+) and regulatory T Cells (Tregs, CD3+CD4+CD25+FoxP3+).[12] Helper T Cells (CD45+CD3+CD4+) assist other immune cells by secreting cytokines that enhance the anti-tumor immune response. Cytotoxic T Cells (CD45+CD3+CD8+) are primarily responsible for directly killing cancer cells. They recognize and bind to antigens presented by tumor cells, leading to the release of cytotoxic granules that induce apoptosis in the target cells. Regulatory T Cells (Tregs, CD3+CD4+CD25+FoxP3+) maintain immune homeostasis and prevent autoimmunity, but in the tumor microenvironment they can suppress effective anti-tumor immune responses, allowing cancer cells to evade immune surveillance.[12,16] There are two subsets of NK cells: $CD56^{bright}CD16^{dim/-}$ and $CD56^{dim}CD16^{+}$. NK cells ($CD56^{bright}CD16^{dim/-}$) are believed to be less mature and a potent cytokine producer, while NK cells ($CD56^{dim}CD16^{+}$) are believed to be more mature and the most cytotoxic.[20,21] In the peripheral blood, more than 85% of NK cells are CD56dimCD16+. However, in tissues, the relative proportion of NK cell subsets can be very different from that in peripheral blood.[22] NK cells are short-lived lymphocytes and have shown high cancer susceptibility in mouse models and clinical studies.[23,24] Compare these lymphocyte subsets' percentages between patients at different stages of their chemotherapy regimen and examine how alterations in lymphocyte subsets may impact clinical management, particularly concerning interventions that promote B cell recovery and maintain immune competence. We hypothesize that there will be discernible patterns in lymphocyte subsets that correlate with the intensity and duration of chemotherapy. Uncovering these patterns could advance our understanding of chemotherapy-induced myelosuppression and inform better management strategies for affected patients.[5]

Material and Methods

Study Design

A retrospective cohort study was conducted to assess the impact of chemotherapy (Taxanes, Pemetrexed, Etoposid, Platinum, Cisplatin, Carboplatin) on peripheral blood lymphocyte subsets in lung cancer patients.

Setting

The study was carried out at The Second People's Hospital of Liaocheng, covering treatments administered from January 1, 2021, to June 1, 2023.

Participants

In this study, we initially reviewed 214 patients, who were diagnosed and treated between January 1, 2021, and June 1, 2023. We refined the selection using the following criteria. Inclusion Criteria: 1. Age between 40 and 73 years; 2. At least two months since the last anti-tumor therapy; 3. ECOG performance status of 0–2; 4. Cancer stages II–IV; 5. Normal organ function. Exclusion Criteria: 1. Presence of significant comorbidities (eg, heart failure, liver dysfunction); 2. Active infections. By applying our inclusion and exclusion criteria, we refined the eligible study population to 159 lung cancer patients, consisting of 108 in the no previous therapy (NT) group and 51 in the prior therapy (PT) group.

Variables

The peripheral blood lymphocyte subsets were detected using Agilent flow cytometer and Agilent NovoExpress software following the manufacturer's instructions. Remove Agilent hemolysin from 2–8°C storage and allow it to reach room temperature. Dilute the hemolysin 10-fold to 1× concentration using room temperature purified water. Pipette 20 μL of antibody reagent from the CD3/CD16+CD56/CD45/CD19 detection kit into a flow cytometry tube. Add 50 μL of well-

mixed anticoagulated peripheral blood to the bottom of the tube, avoiding contact with the tube walls. Gently vortex for 5 seconds. Incubate at room temperature (18–25°C) in the dark for 15 minutes. Add 450 μL of 1× Agilent hemolysin. Gently vortex for 5 seconds, and incubate at room temperature in the dark for an additional 15 minutes. After adding the detection reagent to whole blood, the fluorescently labeled antibodies in the reagent specifically bind to white blood cell surface antigens. In the NovoExpress software, create a FSC vs SSC dot plot to identify the lymphocyte population: T cells (CD45+CD3+, including Helper T Cells (CD45+CD3+CD4+) and cytotoxic T Cells (CD45+CD3+CD8+)), B Cells (CD45+ CD19+), and NK Cells (CD45+CD16+CD56+). As shown in Figure 1, we use CD45 and SSC (side scatter) to plot a scatter diagram, and gate the population that is CD45 strong positive and SSC low, which corresponds to lymphocytes. Within the lymphocyte population, we use CD3 and SSC to plot a scatter diagram, and separately gate the CD3+ and CD3- cells. From the CD3+ cells (T cells) population, we analyze the combination of CD4+ and CD8+ to distinguish helper T cells (TH cells) and cytotoxic T cells (TC cells). From the CD3- cells population, we identify CD19+ cells (B cells) and CD16+CD56+ cells (natural killer cells, NK cells). We consider lymphocytes as a whole and then calculate the percentages of each lymphocyte subsets.

Data Sources/Measurement

Baseline measurements (NT and PT group) were taken before chemotherapy initiation, for part of the patients there are multiple samples, indicating that there are more samples than patients in the NT and PT groups. From the non-treated (NT) group, 128 samples were collected, while the pre-treated (PT) group provided 75 samples. In the subsequent measurements, although we made every effort to collect more samples, some patients still dropped out of the trial for reasons such as discontinuing treatment or seeking treatment at another hospital. Early treatment cycle (EarlyCycle) group patients, during four or less than four cycles of chemotherapy regimen, contributed 118 samples, and those in later stages (LateCycle) group, more than four cycles, gave 51 samples.

Bias

Selection bias was mitigated by applying strict inclusion and exclusion criteria. These criteria were chosen to ensure a homogeneous study group, minimizing the introduction of confounding variables. Through rigorous gating, we control the potential confounding factors such as comorbidities and functional status. However, we acknowledge that some unmeasured confounding factors might still exist. And all patients presenting who met the predefined inclusion criteria during the study enrollment period were approached for participation. Attrition bias was a significant consideration in the design and implementation of our study. To mitigate its impact, we adopted an intention-to-treat (ITT) analysis approach. All patients who initiated the chemotherapy were included in the final analysis, irrespective of their adherence or

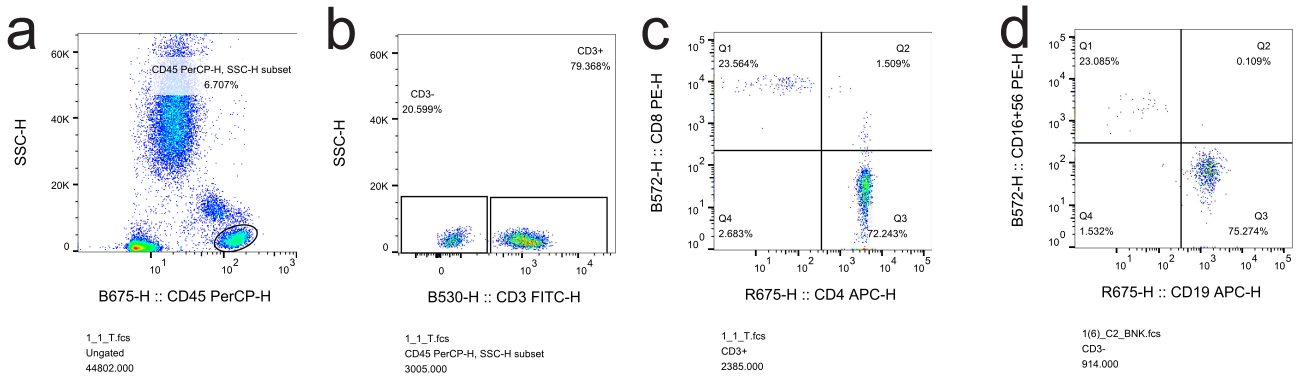

Figure 1 Flow Cytometry. (**a**) Scatter plot showing CD45 PerCP-H versus SSC-H. The cells in cycle are CD45 strong positive and SSC low. (**b**) Scatter plot illustrating CD3 FITC-H versus SSC-H. The left part is CD3- and and SSC low. The right part is CD3+ and and SSC low. (**c**) Scatter plot of CD4 APC-H versus CD8 PE-H, displaying the distribution within CD3+ T cells. The Q1 part is CD3+ and CD8+ cytotoxic T cells. The Q3 part is CD3+ and CD4+ helper T cells. (**d**) Scatter plot of CD19 APC-H versus CD16+56+ PE-H, indicating the distribution of B cells (CD19+) and NK cells (CD16+56+) within CD3- cells. The Q1 part is CD3- and CD16+56+ NK cells. The Q3 part is CD3- and CD19+ B cells.

continuation in the study to its completion. This approach ensures that our findings are more generalizable and not solely reflective of the outcomes in patients who completed the treatment protocol.

Study Size

Utilizing R statistical software (version 4.3.2) with the "pwr" package, we determined our study size.[25] The effect size was set to detect a clinically meaningful difference in lymphocyte subset percentages, pivotal for assessing immune function alterations due to chemotherapy. With an 80% power, a 5% alpha level and a 0.5 effect size.

Quantitative Variables

Median and Interquartile Ranges (IQRs) were used to describe non-normally distributed data.

Statistical Methods

Firstly, since some patients dropped out of the trial, we did not have enough samples compared to the baseline. Therefore, multiple imputation was performed using R's "mice" package with a random forest algorithm for missing values.[26] By employing multiple imputation, we aimed to generate several plausible datasets where the missing values are filled in by predictions based on the observed data. These complete datasets were then analyzed using standard statistical procedures, and the results were pooled to produce estimates that reflect the uncertainty due to the missing data. This method enhances the robustness of our statistical tests and ensures that our analyses are not unduly influenced by the missing data patterns. Then due to data non-normality, which remained after an arcsine square root transformation was applied,[27] the Kruskal–Wallis test assessed differences across groups, with post-hoc comparisons via the Wilcoxon rank-sum test and Bonferroni correction for multiple testing.[28,29] This conservative approach adjusts the p-values obtained from the pairwise comparisons to account for the increased chance of false positives when multiple tests are performed. The entirety of the statistical analysis was executed using R software (version 4.3.2), chosen for its proficiency in handling non-parametric data and advanced statistical methods.

Results

The study included 159 lung cancer patients, with 108 in the no previous therapy (NT) group and 51 in the prior therapy (PT) group. Participants were further grouped into EarlyCycle (≤4 chemotherapy cycles) and LateCycle (>4 chemotherapy cycles) based on their treatment regimen. The baseline demographic and disease characteristics of the participants are reported in Table 1.

The primary outcomes measured were changes in median and mean percentages of lymphocyte subsets, including T cells, CD4+ T cells, CD8+ T cells, B cells, and NK cells.

Table 1 The Baseline Demographic and Disease Characteristics of Participants

Characteristic	Level	Overall
Patients(n)		159
Gender (%)	female	72(45.3)
	male	87(54.7)
Age(median)		62(40–73)
Histology (%)	Adenocarcinoma	75(47.2)
	Squamous Cell Carcinoma	33(20.8)
	Small Cell Lung Cancer	36 (22.6)
	Large Cell (Undifferentiated) Carcinoma	15(9.4)
Stage (%)	II	21(13.2)
	III	48(30.2)
	IV	90(56.6)

For T cells (CD45+CD3+, including Helper T Cells (CD45+CD3+CD4+) and cytotoxic T Cells (CD45+CD3+CD8+)), the EarlyCycle group showed a significant increase in median percentages compared to both NT (0.7783 vs 0.7271; p=0.0017) and PT (0.7783 vs 0.6804; p=1.6e-05). The LateCycle group's median percentages increase was not statistically significant from NT (p=0.1079) but was significant from PT (0.7479 vs 0.6804; p=0.0005). No significant differences were observed within the groups (p=1.00). For CD4+ T cells, there was no significant change in median percentages from NT to EarlyCycle (p=1.00) or LateCycle (p=1.00). However, there was a significant decrease from PT to EarlyCycle (0.4377 vs 0.3687; p=4.7e-06) and a non-significant trend from PT to LateCycle (p=0.42183). For CD8+ T cells, no significant changes in median percentages were observed across any groups, with all p-values equating to 1.00. We can find differences in Figure 2.

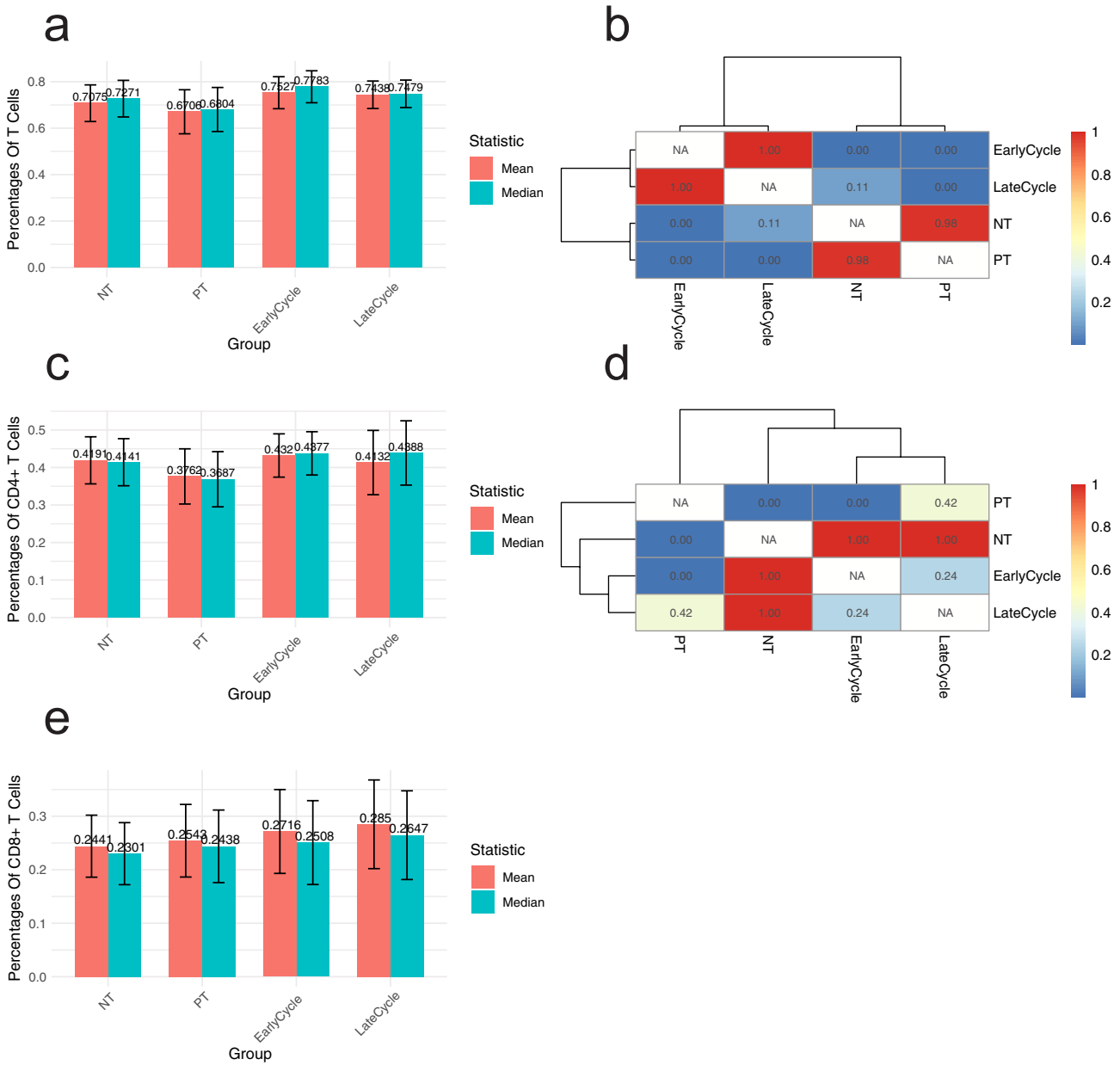

Figure 2 Median and Mean percentages of T cells (including CD4+ T cells and CD8+ T cells). (**a**) Bar chart of Median and Mean percentages of T cells. (**b**) Heatmap of median percentages of T cells show changes between each group. (**c**) Bar chart of Median and Mean percentages of CD4+ T cells. (**d**) Heatmap of median percentages of CD4+ T cells show changes between each group. (**e**) Bar chart of Median and Mean percentages of CD8+ T cells. There are no significant changes between each group of CD8+ T cells, so there is no heatmap of CD8+ T cells. In the bar charts, the Orange is mean and the green is median. In the heatmaps, the dark blue represents a significant change between the two groups and the light blue and red represent no significant changes between the two groups.

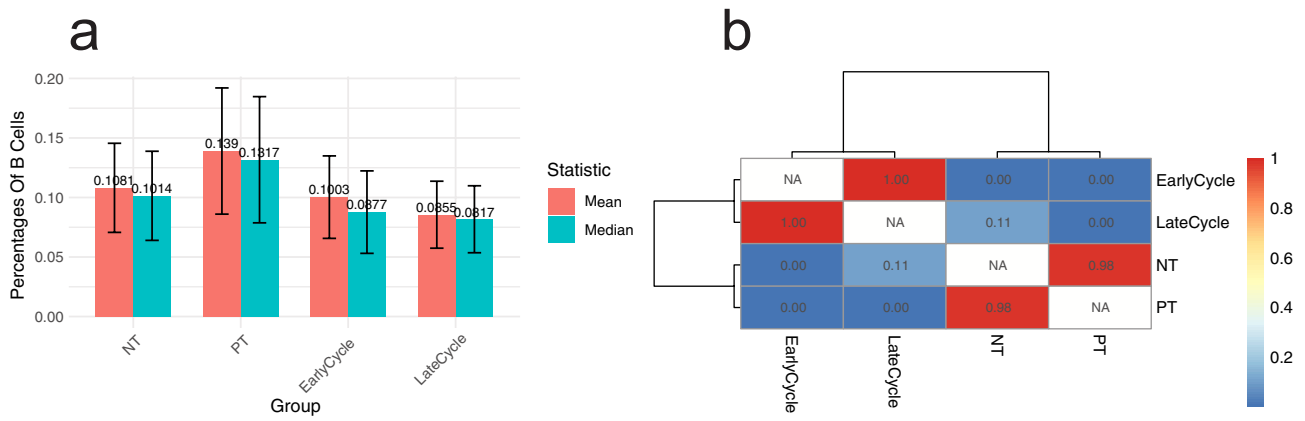

Figure 3 Median and Mean percentages of B cells. (**a**) Bar chart of Median and Mean percentages of B cells. (**b**) Heatmap of median percentages of B cells show changes between each group.

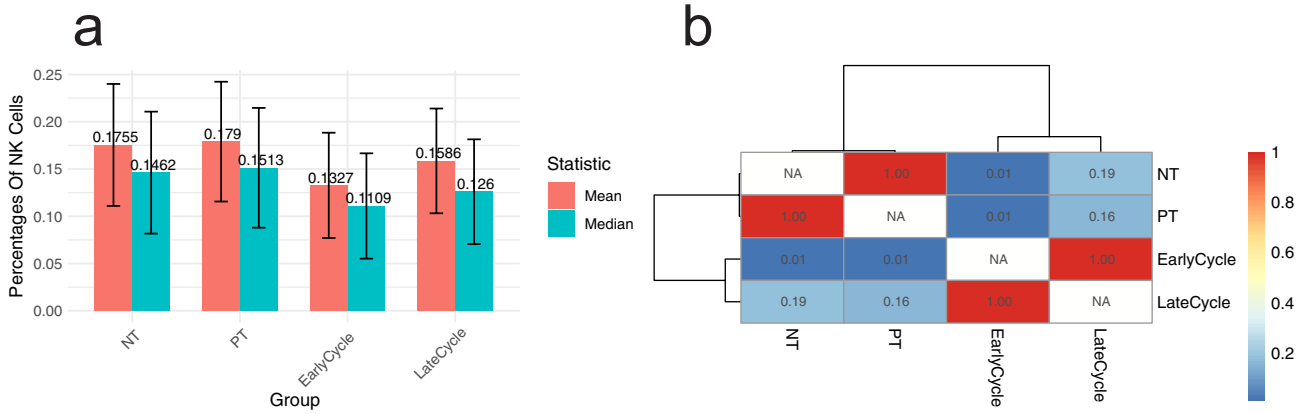

Figure 4 Median and Mean percentages of NK cells. (**a**) Bar chart of Median and Mean percentages of NK cells. (**b**) Heatmap of median percentages of NK cells show changes between each group.

For B cells (CD45+ CD19+), significant decreases in median percentages were observed from NT to LateCycle (0.1014 vs 0.0817; p=2.2e-05) and from PT to LateCycle (0.1317 vs 0.0817; p=6.2e-10). The EarlyCycle group also showed a significant decrease from PT (p=0.00051), but not from NT (p=1). We can find differences in Figure 3.

For NK cells (CD45+CD16+CD56+), significant decrease in median percentages were observed in the EarlyCycle group compared to both NT (0.1109 vs 0.1462; p=0.00816) and PT (0.1109 vs 0.1513; p=0.00992). No significant changes were observed in the LateCycle group. We can find differences in Figure 4.

Discussion

Our study showed alterations in lymphocyte subsets following chemotherapy, revealing significant treatment-phase-dependent immunological shifts in lung cancer patients. The significant elevation in T cells during the early cycles of chemotherapy could be indicative of an initial compensatory mechanism or a selective resistance of certain T cell subsets to chemotherapy-induced myelosuppression.[30–35] This phenomenon aligns with previous reports suggesting that certain cytotoxic agents may spare, or even stimulate, T-cell populations in the short term.

The absence of significant changes in CD4+ and CD8+ T cell percentages is somewhat unexpected, given their central role in antitumor immunity and previous studies reporting their susceptibility to chemotherapy. This might indicate a selective resilience or recovery of these subsets in our patient cohort or could be attributed to the heterogeneity of chemotherapeutic regimens and their differential effects on lymphocyte subsets. Although this study does not focus on regulatory T cells (Tregs), but they play a crucial role in maintaining immune homeostasis and preventing autoimmunity.

However, in the context of cancer Tregs can contribute to tumor progression by suppressing anti-tumor immune responses.[36,37] Recent studies have highlighted the importance of Tregs in chemotherapy resistance and immune escape mechanisms in lung cancer.[38,39] This may be correlated to the elevation in T cells during the early cycles of chemotherapy.

The discernible reduction in B cell percentages observed in patients subjected to more than four cycles of chemotherapy aligns with established knowledge regarding the impact of chemotherapy on humoral immunity.[31,35,40–42] This protracted decrement, evident in the LateCycle group, may be attributable to the relatively extended lifespan and turnover rate of B cells. Alternatively, it could be indicative of a deeper, more sustained suppression of B cell precursors due to cumulative chemotherapy exposure. The clinical ramifications of this phenomenon are significant, potentially predisposing patients to an elevated risk of infectious diseases and attenuating the protective effects of vaccinations. These findings emphasize the critical need for diligent patient management strategies in those receiving multiple chemotherapy cycles. Enhanced monitoring and proactive measures may be necessary to mitigate infection risks ensuring comprehensive care for this vulnerable patient population.

Interestingly, NK cell percentages decreased significantly in the early cycle group but did not change in the late cycle group when compared to NT and PT. Conventionally, there are two subsets of NK cells: $CD56^{bright}CD16^{dim/-}$ and $CD56^{dim}CD16^{+}$. NK cells ($CD56^{bright}CD16^{dim/-}$) are believed to be less mature and a potent cytokine producer, while NK cells ($CD56^{dim}CD16^{+}$) are believed to be more mature and the most cytotoxic. In the peripheral blood, more than 85% of NK cells are $CD56^{dim}CD16.^{+}$[20,21,43] However, in tissues, the relative proportion of NK subsets can be very different from that in peripheral blood.[21,22] NK cells are short-lived lymphocytes and have shown high cancer susceptibility in mouse models and clinical studies. In our study, the significant decrease of peripheral blood NK cells in the early chemotherapy cycles indicates higher susceptibility of NK cells during this period. Researches have shown that in the early stage of anti-tumor therapy, the tumor is more sensitive to chemotherapy, leading to the release of numerous cell fragments into the blood, which subsequently activates and reduces NK cells.[44–47] Combined with these researches, we found that it is the $CD56^{dim}CD16^{+}$ NK subset cells that decrease more than $CD56^{bright}CD16^{dim/-}$ NK subset cells. This means patients lose their mature NK cells, leaving the less mature NK cells during early cycle chemotherapies. The weakened immunosurveillance mediated by NK cells may lead to tumor metastasis. These findings emphasize the critical need for special management strategies to maintain the number and function of NK cells, especially during early chemotherapy cycles. Potential strategies could include the use of cytokine therapy to boost NK cell numbers or the administration of NK cell-stimulating agents to enhance their function.

There are limitations in our study. The retrospective nature limits our ability to establish causality, and potential confounders could not be controlled for. The dataset we have scrutinized is quite heterogeneous and we have not yet distinguished subgroups well based on critical variables such as pathological types and the chemotherapeutic agents administered.

Conclusion

Our findings underscore the complex effects of chemotherapy on immune dynamics in lung cancer patients. The differential impact on lymphocyte subsets over time highlights the importance of immune monitoring and may have implications for patient management. Personalizing chemotherapy regimens by considering their immunomodulatory effects could become an integral part of optimizing treatment strategies for lung cancer.

Acknowledgments

This study has been reviewed by the Ethics Committee of Liaocheng Second People's Hospital. We confirm that informed consent was obtained from the study participants. We confirm that the guidelines outlined in the Declaration of Helsinki were followed.

Disclosure

The authors report no conflicts of interest in this work.

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