Circulating NK and T cell subpopulations correlate with response to immune checkpoint blockade in sarcoma patients

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All glory to God.

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ABSTRACT

Circulating NK and T cell subpopulations correlate with response to immune checkpoint blockade in sarcoma patients

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Sarcomas are rare types of malignancies, and their median survival is 12 months despite classical treatment. Recently, PD-1/PD-L1 inhibitors have been proposed as a new potential therapy. However, due to their unsatisfactory clinical response, screening patients who may have a clinical benefit is essential.

In this study, we performed immune cell profiling in sarcoma patient's PBMCs before treatment with the immune checkpoint inhibitors nivolumab and durvalumab (n=17).

A significantly higher percentage of CD3-CD56+NK cells was observed in PR compared to SD and PD at baseline. In addition, an increased proportion of PD-1 expressing CD4+T cells was associated with positive drug response. However, PD-1 expression in CD8+T cells did not seem to be associated with clinical outcome.

Our results demonstrate that high proportions of NK cells and PD-1 expressing CD4+T cells observed at baseline are associated with better clinical outcomes, and can therefore represent predictive effectiveness in sarcoma patients.

Key words : sarcoma, immune checkpoint inhibitor, predictive biomarker

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I. INTRODUCTION

Sarcomas are rare types of malignancies, reported as approximately 1% of total malignancies. Despite the rarity, sarcomas are heterogeneous malignancies of mesenchymal origin and are classified into more than 100 distinct subtypes[1,2]. Chemotherapy and radiotherapy have been used for the treatment of metastatic sarcoma, but as the median survival of patients is 12 months, the therapeutic outcome of the treatment is insufficient[3-8]. Therefore, several novel treatment options have been studied to overcome the present treatment. Recently, immune checkpoint inhibitors have been suggested as one of the new potential therapeutics. Among them, targeting programmed death 1 (PD-1) or PD-ligand 1 (PD-L1) has improved the clinical outcome in patients of melanoma and other cancers.

The programmed cell death protein 1/programmed cell death-ligand 1 (PD-1/PD-L1) pathway is an important immune checkpoint pathway[9-12]. The PD-1/PD-L1 axis affects the balance between tumor immune surveillance and immune resistance[13-15]. Increased PD-L1 expression on tumor cells or tumor-infiltrated lymphocytes (TIL) results in T-cell exhaustion[16], weakening tumor-specific immunity and promoting tumor progression[13,17]. However, in clinical practice, the main drawback of PD-1/PD-L1 inhibitors is the dissatisfying response rate of cancer patient[13].

Therefore, patient selection for clinical trials must be made prior to PD-1/PD-L1 inhibitor treatment[14,18,19]. In general, the expression of PD-L1 on a tumor and the tumor mutational burden (TMB) are considered indicators for predicting the rate of response to immune checkpoint inhibitors, but their predictions do not often correlate with the actual response rate[20,21]. This is due to the inconsistent distribution of PD-L1+ tumor or stromal cells between biopsy specimens and resection tissue. Also, a single-site biopsy could overrate the level of clonal mutation and cause the poor response of some patients with high TMB[13,22-24].

Tracking the biomarkers in peripheral blood gives more useful and less heterogeneous results than biopsy samples of tumor tissue in treatment strategies[13,22]. As a result, this is the approach taken to optimize treatment strategies.

In this study, we performed immune cell profiling in sarcoma patient's PBMCs before treatment with immune checkpoint inhibitors including nivolumab and durvalumab. To identify prognostic factors for treatment response among the cell subtypes of PBMCs, the proportion of immune cells such as CD4+T cells, CD8+T cells, NK cells, and monocytes and their expression of PD-1/PD-L1 was analyzed. We determined correlations between circulating immune cells and clinical outcome and suggested convincing predictive markers for anti- PD-1/PD-L1 therapy.

II. MATERIALS AND METHODS

1. Patient population and study procedure

PD-1/PD-L1 blockade treated sarcoma patients agreed to have tissue and blood samples collected and stored during their treatment. They gave permission to the review of their past medical records, cancer tumor type, toxicity assessments, clinical response, survival, and laboratory data. Patients received anti–PD-1 therapy nivolumab (3 mg/kg every 2 weeks), or anti–PD-L1 therapy durvalumab (1500 mg/m2) and doxorubicin (75

mg/m2) as an IV infusion for 3 weeks until disease progression or an unacceptable side effect. Tumor size was measured by CT or MRI and evaluated for response using Response Evaluation Criteria in Solid Tumors 1.1 (RECIST 1.1).

Patients were divided into three groups according to the clinical outcome as partial response (PR): at least a 30% decrease in the sum of diameters of target lesions compared with the baseline sum diameters; progressive disease (PD): at least a 20% increase in the sum of diameters of target lesions; stable disease (SD): neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD.

2. PBMC preparation

Peripheral blood was collected in an EDTA-containing tube from sarcoma patients before treatment. Then, the blood sample was layered over Ficoll-Paque (GE Healthcare, Illinois, USA) and centrifuged at 400 g for 40 min. PBMCs were isolated at the interface of the gradient, washed, counted, and cryopreserved in Recovery Cell Culture Freezing Medium for subsequent analysis.

3. Cancer patient derived PBMCs and flow cytometry analysis

Cryopreserved PBMCs were thawed and washed once. Then, the samples were stained with fluorochrome-conjugated antibodies against surface markers for 30 min at 4°C and washed again. For intracellular staining, surface-stained cells were permeabilized using a Foxp3 Staining Buffer Kit (eBioscience, CA, USA) and intracellular markers were stained. Cells were stained with the following fluorophore-conjugated monoclonal antibodies obtained from Biolegend (CA, USA): PE-CD3, FITC-CD8a, APC-Cy7-PD-1, Alexa fluor 700-CD4, BV421-CD152(CTLA4), PerCP-Cy5.5-TBET, BV421-ki67, BV421-CD14, Alexa fluor 700-CD16, and BV605-CD56 as well as PE-eFluor 610-Eomes from eBioscience. Flow cytometry was performed using a SA3800 spectral analyzer (Sony Biotechnology, CA, USA) and the data were analyzed using FlowJo software (BD, New Jersey, USA).

4. Immunohistochemistry and evaluation of PD-L1 expression

PD-L1 expression was measured using tumor samples of patients treated with PD-1/PD-L1 blockade. Biopsies were fixed with formalin, embedded in paraffin, and cut into 5 μm thick sections. The sections were stained using anti-PD-L1 antibody (clone SP263, Ventana), and PD-L1 protein was visualized using an OptiView DAB IHC Detection Kit and an OptiView Amplification Kit (both Ventana). The PD-L1 tumor ratio score (TPS) represents the proportion at which more than 100 viable cells exhibit full or partial membrane staining.

5. Statistical analysis

All values are given as the mean ± standard deviation (SD). Statistical analysis of group differences was carried out using Mann Whitney test. Relation analysis was conducted by Pearson correlation test. A value of $p < 0.05$ was considered statistically significant.

III. RESULTS

1. Clinical outcome of patients treated with PD-1/PD-L1 inhibitor

A total of 17 patients were treated consisting of 12 patients treated with durvalumab and doxorubicin, and 5 patients treated with nivolumab. The clinical outcome was divided into three groups: 1) partial response (PR); 2) stable disease (SD); 3) progression disease (PD) according to response evaluation criteria in solid tumor (RECIST). Based on the above grouping, 5 of the 17 sarcoma patients were in the PR group, 10 were in the SD group, and 2 were in the PD group.

Table 1. Patient population

Index	Number
Age (years of median, range)	54 (32-83)
Histology	
Leimyosarcoma	6
Carcinosarcoma	5
Solitary fibrous tumor	2
Epithelioid sarcoma	1
Malignant peripheral nerve sheath tumor	1
Myofibroblastic sarcoma	1
Spindle cell sarcoma	1
Drug	
Durvalumamb + Doxorubicin	12
Nivolumab	5
Line of treatment	
1	13
2	4
Previous surgical resection	
Curative only	5
Palliative only	5
Both	4
None	3
Previous radiotherapy	
Adjuvant only	4
Both (adjuvant and palliative)	1
None	12
Best response	
Partial response	5
Stable disease	10
Progressive disease	2
PFS[1] (days of median, range)	219 (30~282+)
OS[2] (days of median, range)	Not reached (63+~354+)

[1]progressive- free survival, [2]overall survival

2. Association between PBMC subsets and patient outcomes

The cell distribution and expression patterns of CD markers on immune cells in sarcoma patients were identified by separating PBMCs before anti-PD-1 or anti-PD-L1 drug treatment. The percentages of CD3+CD4+, CD3+CD8+T cells, CD3-CD56+NK cells, CD14+ monocytes, PD-1+, and PD-L1+ cells were analyzed via flow cytometry. Gating strategies of lymphocyte and monocyte subsets are shown in Fig. 2.

Significantly higher percentage of CD3-CD56+NK cells was observed in the PR group (10.40±6.20%) and the SD group (8.70±3.74%) compared to the PD (5.23±00.16%) groups at baseline (PR vs PD : $P<0.0476$, SD vs PD : $P<0.0152$) (Fig. 1A). This result indicates that the percentage of NK cells in PBMCs before treatment is related to the high response rate of the PD-1/PD-L1 blockade. However, PD-1 and PD-L1 expression in NK cells were not distinctive features according to clinical outcome (data not shown).

Next, CD3+CD4+, and CD3+CD8+T cells in the PBMCs were analyzed at baseline. There was no difference in regard to CD4+T cell among three groups (Fig. 1D), while CD8+ T cells tended to be higher in PD and SD groups compared to PR group (Fig. 1E). Interestingly, the proportion of PD-1 expressing CD4+T cells was higher in the PR and SD groups compared to the PD group (PR: 3.33±0.72%, SD: 4.05±1.01%, PD: 2.04±0.17%), but only the SD and PD group results were statistically significant (Mann-Whitney test, SD vs PD : $P<0.0152$) (Fig. 1B). PD-1 expression in CD8+T cells did not seem to be associated with clinical outcome (Fig. 1C).

No association was observed between the percentage of CD14+ monocytes and clinical outcome (Fig. 1F). Representative images of the flowcytometry analysis for the clinical responses are presented in Fig. 1G.

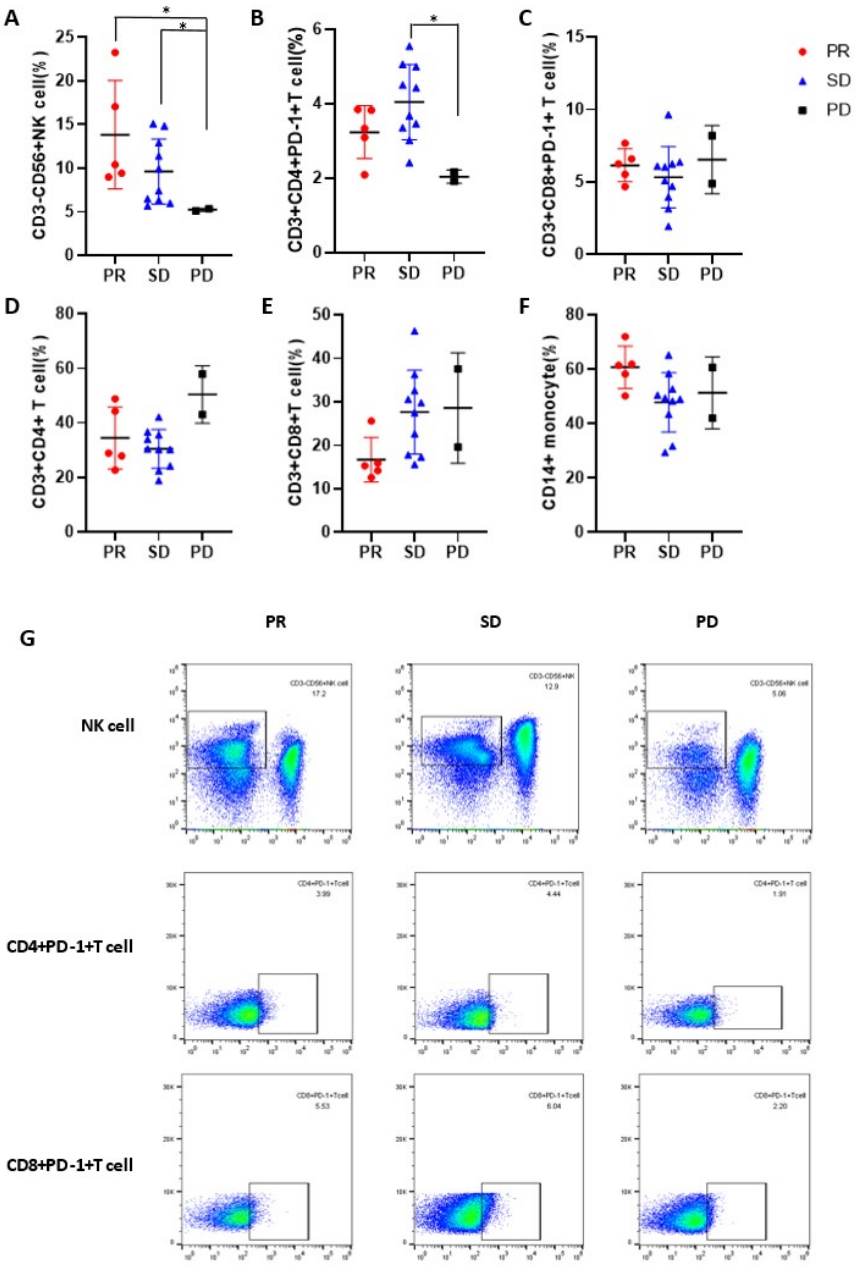

Figure 1. Association between PBMC subsets and patient outcomes. (A) CD3-CD56+NK

cell proportion in PBMC at baseline (partial response (PR) : 10.40±6.20% (mean±SD), stable disease (SD) : 8.70±3.74%, progression disease (PD) : 5.23±00.16%, PR vs PD : P<0.0476, SD vs PD : P<0.0152, PR vs SD : ns, Mann-Whitney test), (B) CD3+CD4+PD-1+T cell proportion (PR : 3.33±0.72%, SD : 4.05±1.01%, PD : 2.04±0.17%, SD vs PD : P<0.0152, PR vs SD : ns, PR vs PD : ns, Mann-Whitney test), (C) CD3+CD8+PD-1+T cell proportion (PR : 6.26±1.13%, SD : 5.57±2.11%, PD : 6.54±2.35%, ns), (D) CD3+CD4+T cell proportion (PR : 34.52±11.34%, SD : 30.48±7.08%, PD : 50.45±10.53%, ns), (E) CD3+CD8+T cell proportion (PR : 16.72±5.12%, SD : 27.65±9.62%, PD : 28.6±12.72%, ns), (F) CD14+monocyte proportion (PR : 60.76±7.83%, SD : 47.8±10.89%, PD : 51.35±13.22%, ns), (G) representative image of flow cytometry analysis.

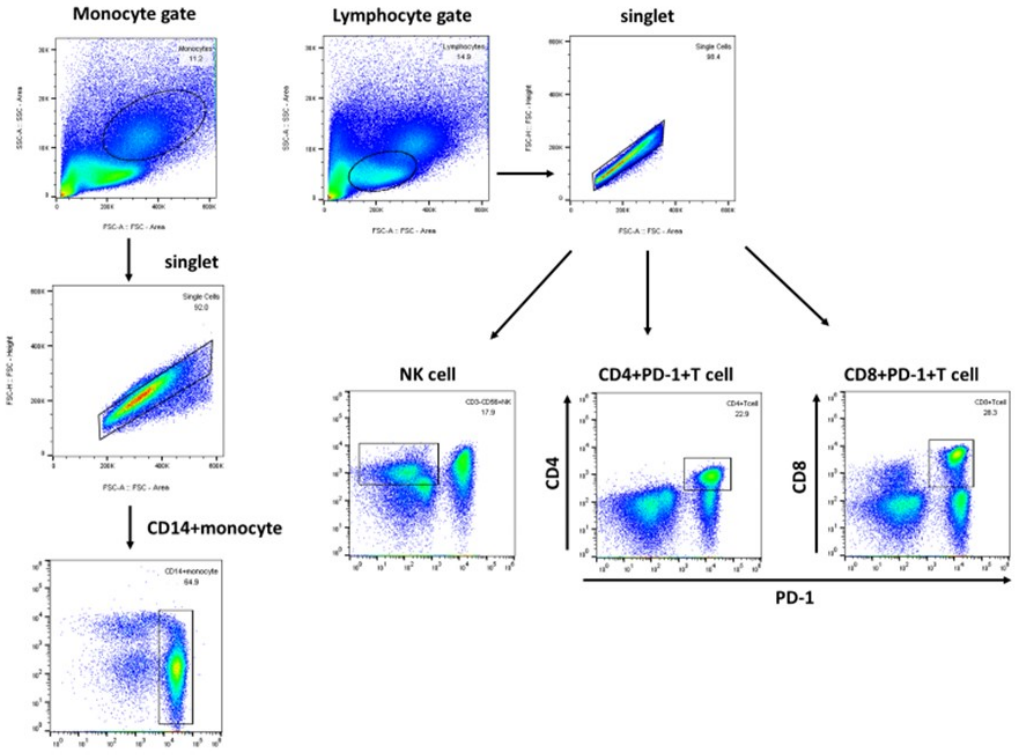

Figure 2. Gating strategy for flow cytometry analysis.

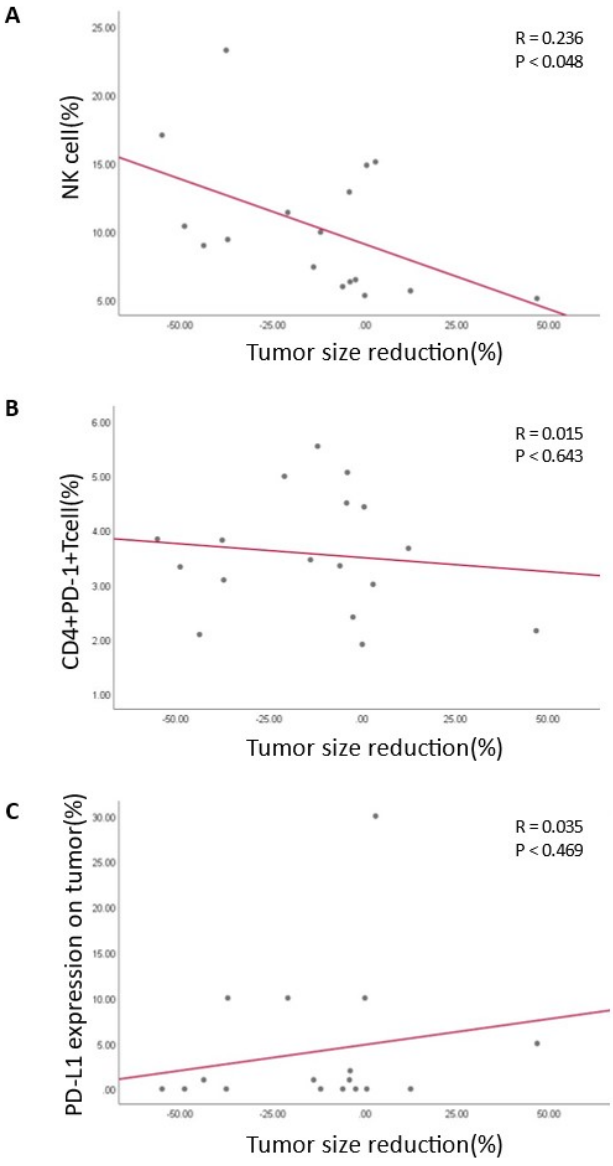

Figure 3. Association between PBMC subsets and tumor size regression. Linear correlation between the percentage of (A) CD3-CD56+NK cell (R=0.236, P<0.048), (B) CD3+CD4+PD-1+T cell (R=0.015, P<0.643), (C) PD-L1 expression on tumor (R=0.035, P<0.469) and maximal tumor size regression. Analysis was performed Pearson test.

3. Association between PBMC subsets and tumor size regression

To identify the association between immune cell subset and the degree of tumor size regression, linear correlation analysis was performed by Pearson test. Percentages of NK cells and CD3+CD4+PD-1+T cells at baseline were inversely correlated to the tumor reduction (Fig. 3A,B), whereas PD-L1 expression on tumor tissue was not associated with tumor regression (Fig. 3C).

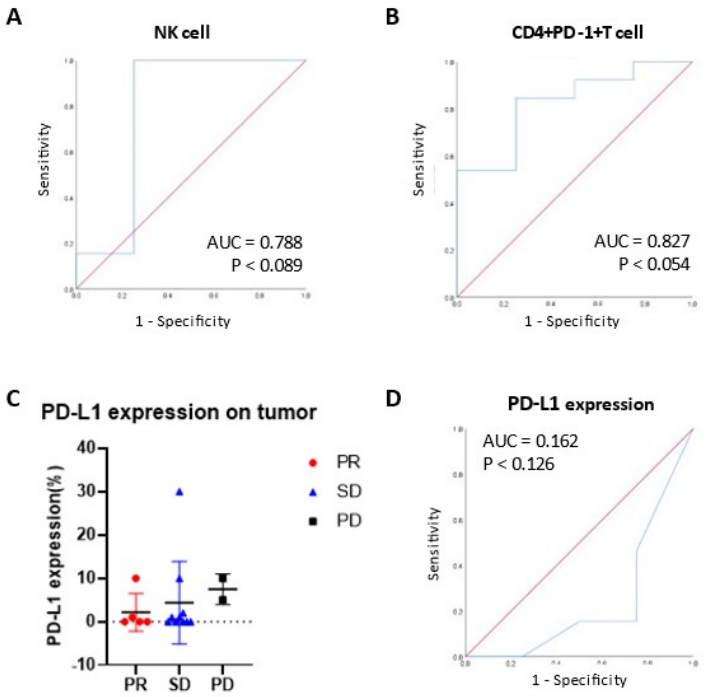

Figure 4. Impact of immune profiles on the response to PD-1/PD-L1 inhibitor. (A) Receiver Operating Characteristic (ROC) curve of the percentage of baseline circulating NK cell (area under the curve (AUC)=0.788,P<0.089), (B) ROC curve of the percentage of baseline circulating CD4+PD-1+T cell (AUC=0.827, P<0.054), (C) the percentage of PD-L1 expression on tumor (PR : 2.2±4.38%, SD : 4.4±9.50%, PD : 7.5±3.53%, ns), (D) ROC curve of the percentage of PD-L1 expression on tumor(AUC=0.162, P<0.126).

4. Impact of immune profiles on the response to PD-1/PD-L1 inhibitors

As we have observed above, patients who have increased proportions of NK cells and CD3+CD4+PD-1+T cells are associated with better clinical outcomes to PD-1/PD-L1 inhibitors treatment. We then analyzed the ROC curves and AUC to validate these cells as predictors. Each ROC curve was assessed in accordance with relevance between the percentage of immune cell portion and response which was distributed to responder and non-responder groups. Responder group included patients with reduced tumor size, and non-responder group included patients with increased tumor size. For NK cells, AUC=0.788, P<0.089, and for CD4+PD-1+T cells, AUC=0.827, P<0.054, which indicate these cells are positive biomarkers (Fig. 4A,B). On the other hand, the expression of PD-L1 on tumor commonly used prognostic marker was not associated with the clinical response and the results of the ROC curve also produced an AUC value of 0.162, P<0.126 (Fig.4C,D). This implies that tumor PD-L1 is limited in its use as a predictive factor.

IV. DISCUSSION

As CD8+ effector cytotoxic T cells (CTLs) are crucial in cancer cell removal, the major goal of cancer immunotherapy is boosting T-cell specific immune responses[9]. Thus, the number of CD8+T cells infiltrating the tumor and PD-L1 expression in TME have been suggested as biomarkers for immunotherapy responses[9,25]. However, the clinical implications of the predictability of each immune cell subset in peripheral blood are unclear especially in sarcoma.

Controversial results have been reported for the evaluation of immune profiling, which can predict the efficacy of immune checkpoint blockade on clinical outcomes[21,26-29]. Comprehensive analysis of immune cell subsets in advanced NSCLC patients revealed that the proportion of cytotoxic lymphocyte expressing PD-1 and circulating NK cells at

baseline can help in identifying patients who are more likely to experience effective treatment with a PD-1 inhibitor[26]. In melanoma patients who received anti-PD-1 therapy, the number of NK cells in peripheral blood was functionally associated with drug response[26,30,31]. As well as NK cells, a gradual increase in the subset of memory CD4+T cells has been reported to predict continuous clinical benefits in melanoma patients receiving anti-PD-1 drugs[26,32]. These results indicate that these cells play a leading role in immune monitoring of cancer patients.

Therefore, we collected peripheral blood from sarcoma patients before PD-1/PD-L1 targeted therapy (n=17) and analyzed the distribution of circulating immune cells and their PD-1 expression to determine the association between drug response and immune cell subset. PR and SD groups had a high proportion of NK cells, as well as high PD-1 expression in CD4+T cells. On the other hand, the PD group had a low percentage of NK cells as well as low PD-1 expression in CD4+ T cells. These NK cell results are in agreement with other studies in NSCLC patients[26]. The percentage of NK cells and CD4+PD-1+T cells correlate with the degree of tumor size regression, as well as the association with the three groups (PR, SD and PD) divided by the clinical outcome. These results suggest that NK cells and CD4+PD-1+T cells are valid as predictive markers in PBMCs in sarcoma patients.

Because the immune checkpoint inhibitors, including anti-PD-1, restore the T cell response that was suppressed by the PD-1 pathway, inhibitors can work more effectively when PD-1 is expressed on T cells[33]. This hypothesis seems to be a possible mechanism for our results which show high expression of PD-1 on CD4 T cells in sarcoma patients with better response to anti-PD-1/PD-L1. Similarly, another research group has reported that responders had higher expression of PD-1 on CD4+ T cells than non-responder in advanced cancer patients[34].

Cancer immune editing and surveillance is mostly systematic, with effector phenotypes (CD8+T cells, CD4+T cells, NK cells) exerting an adverse effect on the development of cancer in tissue and circulatory compartments[26,35]. Other studies have

examined these indicators in NLCLC patients and reported that patients with circulating cells having anti-tumor potential are more likely to respond to anti-PD-1 treatment[26]. Because NK cells recruit dendritic cells in tumor sites and play a leading role in cancer immune surveillance[26,36], our observation of a high proportion of NK cells being associated with better clinical outcome suggests that NK cells can be considered a predictor about response to immune checkpoint inhibitors.

Although PD-L1 expression has been considered a predictor in many clinical trials, the consequences have not been constant[13]. In this study, we also determined that there is no significant correlation between PD-L1 expression on tumors and clinical outcome. There are several reasons that can explain this phenomenon. First of all, different cutoff values and scoring methods for immunohistochemistry (IHC) to assess PD-L1 expression can cause discordance with results[19,37,38]. Furthermore, various causes such as intracellular oncogenic variations or presentation to TIL-derived cytokines can contribute to increased PD-L1 expression[39]. Finally, the real state of PD-L1 can be misinterpreted because of intra-tumoral heterogeneity and dynamic alteration of PD-L1 expression[40,41].

There have been some reports that high PD-1 expression in CD8+T cells in PBMCs or TILs correlate with positive clinical outcomes[42]. In this study, however, CD8+PD-1+T cell expression did not correlate with clinical results. As a reason for these results, we suppose that CD8+T cells act directly on the tumor cell so most CD8+T cells may infiltrate into the tumor site and only a few remaining CD8+T cells circulate in the peripheral blood. To confirm this phenomenon, further study is required to identify the association between the percentage of infiltrating CD8+T cells and circulating CD8+T cells depending on the presence of a tumor.

Even though the sample size of this study was relatively small, this is the first study to report the prognostic significance of the distribution of various immune cell subsets in circulating PBMCs in sarcoma patients.

V. CONCLUSION

In conclusion, this study shows the prognostic potential of patient PBMC subsets before immune checkpoint inhibitor treatment by assessing the correlation between clinical outcome and immune cell profiling in PBMCs. The most outstanding feature of our results is that high proportions of NK cells and PD-1 expressing CD4+T cells observed at baseline are associated with better clinical outcomes, and can therefore represent predictive effectiveness in sarcoma patients.

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ABSTRACT(IN KOREAN)

육종암 환자에서 순환하는 NK세포와 CD4+PD-1+T cell의 발현으로 PD-1/PD-L1 저해제에 대한 치료결과를 예측 연구

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김수정

육종암은 그간 고전적인 치료가 시도되었음에도 불구하고 생존 기간의 중간값이 12개월 밖에 되지 않는 희귀 암종이다. 최근 PD-1/PD-L1 저해제가 가능성이 있는 새로운 치료제로 떠오르고 있다. 그러나 치료 효과가 항상 만족스럽지만은 않기 때문에 치료 전 어떤 환자가 임상적으로 좋은 결과를 얻을 수 있을지 선별하는 것이 중요하다.

이 연구에서는 면역관문 억제제인 니볼루맙과 더발루맙을 투약하기 전, 육종암 환자의 말초혈액 단핵구 세포를 분리하여 면역세포 프로파일링을 실시하였다.

PD 환자군에 비하여 PR과 SD 환자군에서는 CD3-CD56+NK세포 비율이 상당히 높은 것으로 관찰되었고, CD4+T세포에서의 PD-1발현 또한 약제에 대한 반응성이 좋은 환자군에서 증가되어 있는 것으로 나타났다. 하지만 CD8+세포에서의 PD-1발현은 치료 결과와 연관성을 보이지 않았다.

이러한 결과는 치료 전 환자의 말초혈액에서 높은 NK세포의 비율과, CD4+세포에서의 증가된 PD-1발현이 더 좋은 치료 결과와 연관성이 있음을 보여주며, 이는 육종암 환자에서 효과적인 예측 바이오마커가 될 수 있음을 시사한다.

핵심되는 말 : 육종암, 면역관문 억제제, 예측 바이오마커