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Advanced NSCLC patients with high IL-6 levels have altered peripheral T cell population and signaling

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ABSTRACT

Objectives: High levels of circulating interleukin-6 (IL-6) are associated with a poor prognosis in many types of cancer including non-small cell lung cancer (NSCLC). While the inflammatory cytokine can stimulate the immune system and promote tumor growth, it remains unclear how circulating IL-6 can potentiate a poor prognosis. We hypothesized that a mechanism for IL-6-associated poor prognosis is that these patients would have altered T-cell populations and impaired T-cell signaling.

Materials and methods: Plasma levels of IL-6 were measured using a Cytometric Bead Array. T-cell populations from Non-small cell lung cancer patients were characterized using surface markers by flow cytometry, and signaling in the T-cell populations were measured by PhosFlow cytometry.

Results: We determine that patients with high circulating IL-6 levels had distinct T cell characteristics relative to those with low levels. Patients with high levels of IL-6 had significantly more T_{reg} cells and elevated Programmed cell death protein-1 (PD-1) expression on $CD4^+$, $CD8^+$, Treg, and Th17 cells. These patients also showed impaired signal transducer and activator of transcription-1 (STAT1) signaling upon stimulation with IL-6 and phorbol 12-myristate 13-acetate (PMA), and T-Cells from a healthy donor that were treated for four days with IL-6 displayed a similar muting of STAT signaling, which verified the effect seen in patient samples.

Conclusions: This work directly links circulating IL-6 with other poor prognostic indicators, STAT1 and PD-1, and highlights the effects of circulating IL-6 on the immune system. Our data suggest that alteration in T cell populations and function may be a mechanism underlying the poor prognosis seen in NSCLC patients with high IL-6 levels.

1. Introduction

Modulation of the immune system through programmed cell death protein-1 (PD-1) inhibitors, i.e. nivolumab and pembrolizumab, can provide impressive clinical responses in non-small cell lung cancer (NSCLC) patients [1]. Blocking interactions between the inhibitory PD-1 on T cells with its ligand PD-L1 on the tumor or antigen presenting cells can reverse T-cell exhaustion and enhance tumor regression [2]. The striking results seen with checkpoint inhibitors highlights the importance of the immune system in treating cancer.

Aspects of the immune system can contribute to tumor growth and development. High levels of circulating IL-6 is associated with a poor prognosis in lung cancer patients [3]. IL-6 signaling is propagated through the STAT1/3 pathways [4]. Activation of the signal transducer and activator of transcription (STAT) and extracellular signal-regulator Kinase (ERK) pathways in peripheral blood cells can produce anti-tumor activity, and defective STAT1/3 signaling in peripheral blood T cells has been suggested as a poor prognostic indicator in breast cancer [5].

Although a variety of demographic features have been associated with high levels of circulating IL-6, i.e. age, smoking history, and chemotherapy, it remains unclear whether circulating IL-6 is a cause or a consequence of poor survival because possible mechanisms have not been elucidated [6]. Stromal and tumor-derived IL-6 can enhance the survival and proliferation of tumor cells, however, it is not known how circulating IL-6 could affect tumor populations. We proposed that a possible mechanism could be that circulating IL-6 may alter T cell populations or signaling to suppress the immune system and ultimately support tumor growth. We explored this possible relationship by characterizing T cell populations and signaling in advanced NSCLC patients with high and low circulating IL-6 levels.

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2. Materials and methods

Patients who provided written informed consent to participate in an IRB-approved protocol were included in the study. Inclusion criteria included; stage IIIB-IV non-small cell lung cancer, beginning any line of therapy, measurable disease, and ability and willingness to sign an informed consent form. Peripheral blood mononuclear cells (PBMCs) from consented patients were isolated using CPT Cell Preparation Tubes (BD362761). PBMCs from a healthy donor were purchased from ZenBio (Research Triangle Park, North Carolina). Plasma IL-6 levels were measured by flow cytometry using the Cytometric Bead Array (CBA) Th1/Th2/Th17 Cytokine Kit (BD Biosciences, San Jose, CA) according to the manufacturers' instructions. T cell populations and signaling were measured by flow cytometry and analyzed with statistical methods as described in the Supplemental Data. Patient characteristics for the populations and signaling studies are reported in Table S1.

3. Results

3.1. Characterizing the effect of high plasma IL-6 on T cell populations

We measured the circulating IL-6 levels in 70 patients with advanced NSCLC. Patients with high levels of circulating IL-6 (greater than the median, 6.41 pg/ml) exhibited a shorter overall survival than those with low IL-6 levels, 215 versus 707 days ($p < 0.001$), respectively (Fig. S1). To better understand why circulating IL-6 has an adverse effect on survival, we characterized the PBMCs of patients with high and low IL-6 levels. We selected 23 patients with the highest and the 23 patients with the lowest circulating IL-6 levels from our original population and measured various T cells populations in PBMCs by flow cytometry. Five and nine patients from the high and low IL-6 groups, respectively, were excluded from the analysis due to poor viability of the thawed cells for flow cytometry.

The proportions of total $CD8^+$ ($CD3^+$, $CD8^+$), total $CD4^+$ ($CD3^+$, $CD4^+$), T_{h22}, T_{h17}, and T_{fh} T-cells present in the PBMC populations were not significantly different between the IL-6 high and low groups, while the T_{reg} cell population was significantly higher ($p = 0.0273$) in the IL-6 high group (Fig. 1A and C). We also measured the PD-1 expression on each cell type and found that PD-1 expression (determined by median fluorescence intensity, MFI) was significantly higher in the IL-6 high group for $CD8^+$, $CD4^+$, T_{reg}, and T_{h17} cells (Fig. 1B and D).

3.2. High-levels of IL-6 alters intracellular signaling responses in peripheral blood T-cells

We hypothesized that high levels of circulating IL-6 could alter signaling responses in T-cells, therefore, we developed a phosphorylated-protein flow cytometry (PhosFlow) assay to measure changes in phosphorylated STAT1 (pSTAT1; Y701), STAT3 (pSTAT3; Y705), and ERK1/2 (pERK; T202/Y204) upon T-cells stimulation with IL-6 and PMA in T-cells. We measured the signaling responses to IL-6 and PMA in PBMCs from the patient cohorts with low and high circulating IL-6 levels. In this experiment, patient-derived PBMCs were split and either stimulated with IL-6/PMA or left unstimulated. Patients with high levels of circulating IL-6 produced a significantly lower pSTAT1 response than those with low levels, in $CD4^+$ cells ($p = 0.0291$) and $CD4^+$/$CD45RA^-$ cells ($p = 0.0233$), while a similar, but insignificant, trend was seen for Naïve/Effector T cells ($p = 0.0573$) cells upon stimulation (Fig. 2). No significant differences in pSTAT3 and pERK1/2 signaling were observed between the two groups (Fig. 2). The muted pSTAT1 response was due to a significant reduction of the pSTAT1 signal in the IL-6 high cells upon stimulation, as there were no differences in the pSTAT1 levels between the low and high groups without stimulation (Fig. S2). The entire dataset is summarized in Table S4.

We performed an in vitro experiment to confirm that constant high levels of IL-6 attenuates STAT signaling in T-Cells. PBMC from a healthy donor were cultured for 4 days in growth media, or growth media supplemented with 100 pg/ml IL-6, a concentration representing the midpoint of the IL-6 range seen in our original patient cohort, to mimic a low and high IL-6 environment, respectively. PBMCs from both treatment groups were split and either stimulated with IL-6/PMA or left unstimulated (Fig. S2A). PBMCs subjected to IL-6 for four days generated a weaker signaling response through STAT1, STAT3, and MAPK1/2 than that of the control cells upon stimulation for all the T cell

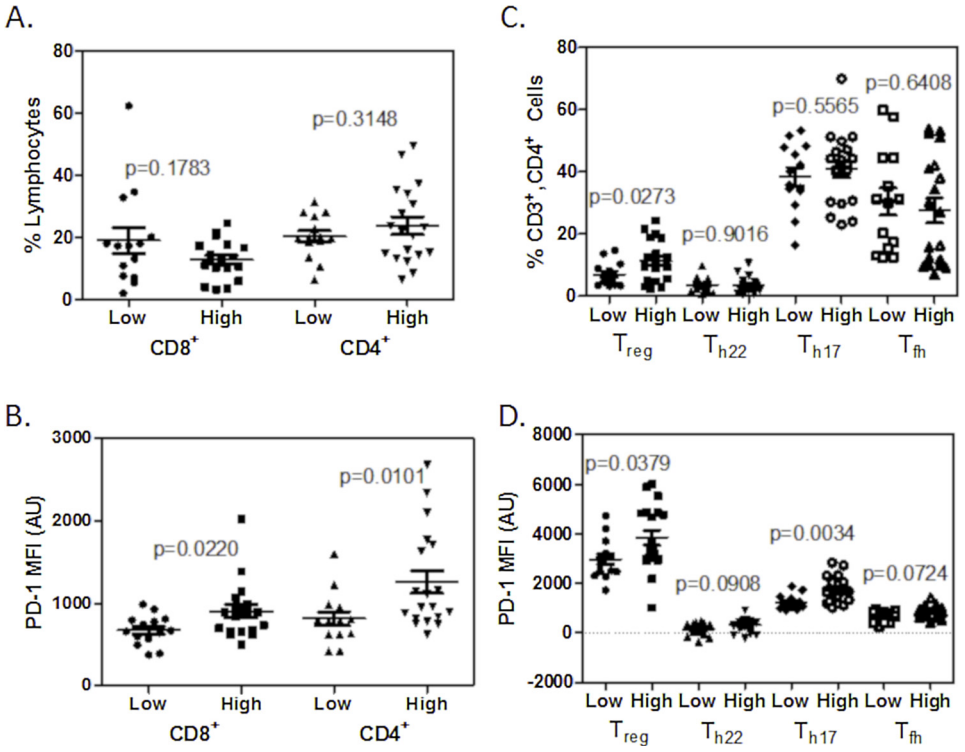

Fig. 1. Characterization of T Cells in patients with low and high levels of IL-6. PBMC were stained with surface markers and subjected to flow cytometry to measure T-cell populations. A–B The proportion of $CD8^+$ and $CD4^+$ cells and the PD-1 expression level (median fluorescent intensity, MFI) for each patient with low or high IL-6. C–D The proportion of various $CD4^+$ populations and the PD-1 expression for each population. Mean and standard error from the mean (SEM) plotted on all graphs.

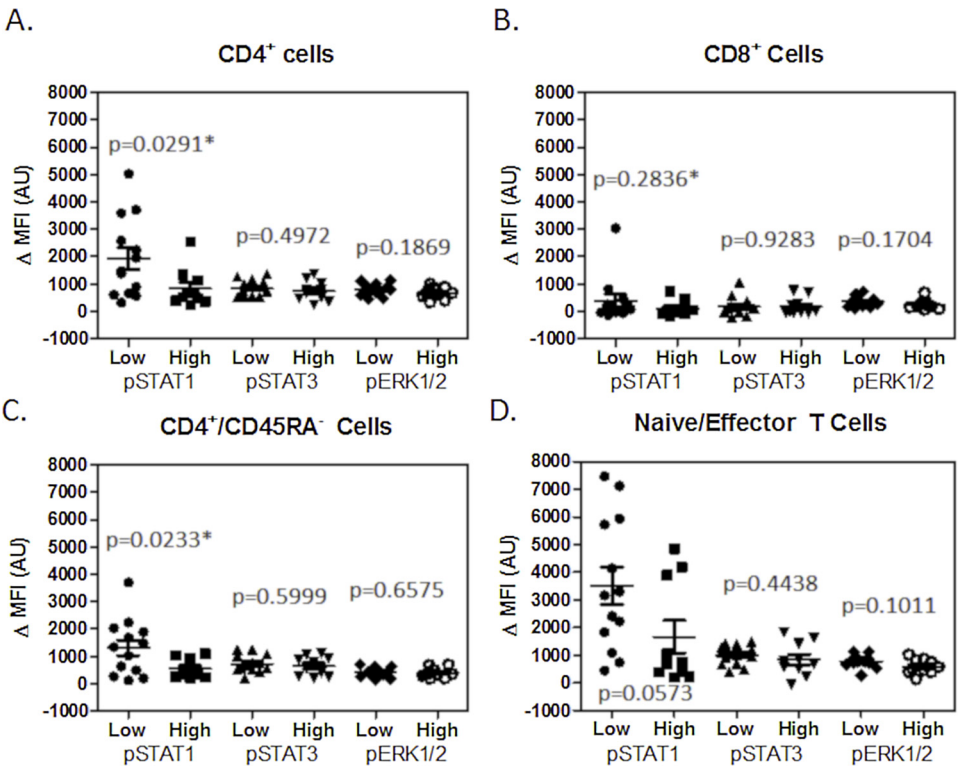

Fig. 2. Characterization of T-cell signaling upon stimulation in patients with low and high levels of IL-6. PBMCs from patients were split, stimulated or not with IL-6/PMA, and subjected to PhosFlow analysis. A–D Samples were compared by the difference between MFI of the stimulated sample and the un-stimulated sample (ΔMFI) for each of the cell populations. Mean and standard error from the mean (SEM) is shown on each graph. Activated and Naïve cells are CD45 positive and negative, respectively. * indicates p-values calculated using a T-Test with a Welsh's correction for unequal variance.

populations measured, $CD4^+$ ($CD4^+$,$CD3^+$), naïve/effector T cells ($CD4^+$,$CD3^+$,$CD45RA^+$), and $CD4^+/CD45RA^-$ T cells ($CD4^+$,$CD3^+$,$CD45^-$; Fig. S3). These results confirm those of the patient samples.

4. Discussion

Circulating IL-6 has frequently been associated with a poor prognosis in cancer patients, and while IL-6 has been linked to various demographic and behavioral characteristics, i.e. age, smoking history…, it remains unclear whether circulating IL-6 directly affects survival or if it is a consequence of poor survival [6]. We characterized T cell populations and signal in patients with high levels or circulating IL-6 and determined that these patient have altered features relative to those with low levels of circulating IL-6. We found that NSCLC patients with low levels of circulating IL-6 had T cell characteristics, PD-1 expression and STAT1/STAT3 responses, similar to those reported for normal donors, while those with high circulating IL-6 had markers associated with poor prognosis including elevated PD-1 expression and muted STAT1 signaling [7]. Those patients in with the poor prognostic indicators, PD-1 expression, and STAT1 signaling, were those that had the shortest survival in our cohort.

The changes we observed in patients with high circulating IL-6 are associated with immune suppression and poor prognosis in cancer patients. Lymphocytes from tumor-bearing mice were found to have muted *Stat1* and *Stat3* phosphorylation compared to those without tumors, which was related to a decrease in immune function in these animals [8]. STAT1 is also required for T cell clonal expansion [9]. T-cell expression of PD-1 can inhibit the immune response and is associated with poor survival in NSCLC patients [7]. Our results suggest that T cell-suppression in a possible mechanism for how high levels of circulating IL-6 can promote tumor progression. We were able to directly link the poor survival associated with high levels of circulating IL-6 to impaired STAT1 signaling and PD-1 expression to suppress the immune system.

This work highlights the effect that circulating IL-6 has on peripheral blood T cells, however, the tumor microenvironment may also contribute to disease progression and shorter survival. Tumor progression can stimulate immune and stromal cells to secrete IL-6 [10]. Additionally, IL-6 can be secreted directly by tumor cell [11]. Regardless of the source, IL-6 can facilitate tumor proliferation and immune invasion [11]. We believe the alterations seen in the peripheral blood T cells are also present in the tumor, and, therefore, IL-6-associated T cell dysfunction may occur in the tumor, which could promote tumor progression and poor survival [12].

This study is limited by the sample size which was affected by the numbers and viability of the PBMC samples used in the study. Additionally, our groups were selected based on circulating IL-6 levels, therefore, correlations between IL-6 levels and an experimental variable could not be determined.

Contributions

SJR: Study design, data analysis, data interpretation and manuscript writing; XL and JZ: Study design, data collection, data interpretation and manuscript editing; BJ: Data collection and data analysis; HZ: Study design, data interpretation and manuscript editing; CPB: Study design, data interpretation, and manuscript editing.

Competing interests

None declared.

Ethics approval

Institutional Review Board of the Penn State College of Medicine.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2019.03.014.

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