Programmed death ligand 1 is overexpressed by neutrophils in the blood of immunocompromised human immunodeficiency virus-negative patients with *Pneumocystis jirovecii* pneumonia

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Despite recent advances in antibiotic use, increasing numbers of human immunodeficiency virus (HIV)-negative patients with *Pneumocystis jirovecii* pneumonia (PCP) are being admitted to intensive care units (ICUs), and show a mortality rate of over 50%.[1] A high neutrophil count in bronchoalveolar lavage (BAL) fluid is a predictor of poor prognosis in HIV-negative patients with PCP.[2] Recently, programmed death ligand 1 (PD-L1) has been identified on neutrophils.[3] However, the role of PD-L1-expressing neutrophils has not been investigated in PCP. Therefore, the purpose of the present study was to examine the expression of PD-L1 on circulating neutrophils and to evaluate their clinical relevance in patients with PCP.

This study was performed in the Department of Respiratory and Critical Care Medicine at the China-Japan Friendship Hospital between September 2017 and October 2018. A total of 17 patients with PCP were recruited. A further ten patients were identified during the diagnostic procedures as being PCP-free not having PCP and were included as controls during the same period. All patients enrolled in the study were HIV-negative, immunocompromised, and with immunosuppressive host conditions, as previously reported.[4] Diagnosis of PCP was based on real-time PCR analysis and Giemsa and methenamine silver staining of BAL fluid samples.

The expression of PD-L1 and programmed death 1 (PD-1) was quantified in peripheral blood cells by flow cytometry. Anti-CD3, anti-CD4, anti-CD8, anti-PD-1, anti-PD-L1, anti-CD16, and anti-CD62L antibodies specific for human proteins were purchased from BD Biosciences (San Jose, CA, USA) and matched isotype controls were used. Red blood cells were lysed using an erythrocyte lysis solution (BD Biosciences). Finally, cells were analyzed using an LSRII Fortessa cytometer (BD Biosciences). FlowJo 10 software (FlowJo, Ashland, OR, USA) was used for data analysis.

Data were analyzed using SPSS 16.0 software (IBM, Chicago, IL, USA). Data are presented as either mean ± standard deviation or median values with an interquartile range (25%, 75%). For continuous variables, the Student's *t* test or Mann-Whitney *U* test was used to assess the differences between two groups. The Fisher exact test was used to compare discrete dichotomous data between two groups. Correlations were assessed using Pearson or Spearman correlation analysis, as appropriate. A significant difference was defined as a *P* value less than 0.05.

Clinical characteristics of the study populations are summarized in Table 1. There was no significant difference found in age or sex ratios between the PCP and control groups. Within the PCP cohort, the most common underlying immunosuppressive disease was interstitial lung disease, followed by systemic disease, hematological malignancy, solid organ transplantation, and solid tumor. No significant differences were observed in either the underlying disease, pulmonary coinfection (including

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Table 1: Characteristics of patients.

Characteristics	Non-PCP ($n = 10$)	PCP ($n = 17$)	*P* value
Age (years), mean ± SD	64.3 ± 12.74	59.47 ± 12.02	0.333
Sex, male, *n*	8	8	0.124
Underlying diseases, *n*			
Solid tumor	1	1	0.999
Hematological malignancy	1	3	0.999
Solid organ transplantation	1	2	0.999
Systemic disease	5	6	0.687
Interstitial lung disease	2	9	0.124
Pulmonary coinfection, *n*			
CMV	4	6	0.999
Bacterial	3	5	0.999
Fungal	0	2	0.516
Immunosuppressive agents use, *n*			
Corticosteroids	7	15	0.326
Anti-tumor chemotherapy	1	3	0.999
T cell immunosuppressant	3	12	0.057
White blood cells ($\times 10^9$/L), median (IQR)	6.56 (6.09–7.95)	11.07 (7.91–12.85)	0.046
Neutrophils ($\times 10^9$/L), median (IQR)	6.12 (4.96–7.19)	9.63 (6.88–10.92)	0.032
$CD4^+$ (cells/μL), median (IQR)	194.00 (150.75–304.05)	158.00 (79.00–236.00)	0.292
$CD8^+$ (cells/μL), median (IQR)	205.50 (114.75–305.00)	223.00 (113.00–384.00)	0.999
APACHE II score, mean ± SD	16.00 ± 4.76	21.47 ± 7.46	0.048

Continuous variables are presented as median with IQR (25%, 75%) or mean ± SD and other values are presented as numbers. Statistical analyses were performed using the Student *t* test, Mann-Whitney *U* test, or Fisher exact tests. $P < 0.05$ was considered statistically significant. APACHE: Acute physiology and chronic health evaluation; CMV: Cytomegalovirus; IQR: Interquartile range; PCP: *Pneumocystis jirovecii* pneumonia; SD: Standard deviation.

cytomegalovirus, bacterial, and fungal coinfection), or the use of immunosuppressive agents between the two cohorts. Notably, there were significantly higher levels of circulating white blood cells and neutrophils in patients with PCP compared with the control group. The disease severity of the PCP group was significantly higher than that of the non-PCP group as determined using the acute physiology and chronic health evaluation (APACHE) II score ($P = 0.0484$).

PD-L1-expressing neutrophils were identified in the peripheral blood by detection of CD11b and PD-L1 using flow cytometry. A representative flow cytometry plot is shown in Figure 1A. Interestingly, peripheral blood neutrophils in the PCP group appeared to have increased levels of PD-L1 compared to those in the control group ($P = 0.0087$; Figure 1B). Subsequently, we found that circulating $CD4^+$ T cells, but not $CD8^+$ T cells from patients with PCP expressed significantly higher levels of PD-1 than those from controls ($P = 0.0053$; Figure 1C and 1D). Taken together, these data suggest that an increased frequency of PD-$L1^+$ neutrophils and PD-1^+/$CD4^+$ T cells is present in patients with PCP.

We next profiled the phenotypes of neutrophil subsets as previously reported.[5] As shown in Supplementary Figure S1A, http://links.lww.com/CM9/A38, using flow cytometry, circulating neutrophils could be classified into three different subsets: mature ($CD62L^{bright}$ $CD16^{bright}CD11b^{bright}$); activated, ($CD62L^{dim}CD16^{bright}$ $CD11b^{bright}$); and immature, ($CD62L^{bright}CD16^{dim}$ $D11b^{bright}$) subsets. No significant differences were observed in the percentages of any of these three neutrophil subsets between PCP and control groups, indicating that patients with PCP do not have an imbalance in circulating neutrophil subsets [Supplementary Figure S1B–D, http://links.lww.com/CM9/A38]. Intriguingly, these data showed an increased level of PD-L1 on activated neutrophil subsets in the PCP group compared to those in the control group ($P = 0.0341$) [Supplementary Figure S2B, http://links.lww.com/CM9/A38], despite their relatively low abundance in the entire neutrophil population. In both groups, neither mature nor immature subsets expressed significant levels of PD-L1 [Supplementary Figure S2C and S2D, http://links.lww.com/CM9/A38].

We found a significant positive correlation between PD-L1 expression on neutrophils and PD-1 expression on $CD4^+$ T cells in patients with PCP ($R = 0.549$, $P = 0.0244$) [Supplementary Figure S3A, http://links.lww.com/CM9/A38]. Interestingly, a significant correlation was also found between the accumulation of PD-L1-expressing activated neutrophil subsets and $CD4^+$ T-cell immunosuppression ($R = 0.4869$, $P = 0.0474$) [Supplementary Figure S3B, http://links.lww.com/CM9/A38]. In particular, there was a clear, positive association between the frequency of PD-$L1^+$ neutrophils and APACHE II scores in patients with PCP ($R = 0.5$, $P = 0.0410$) [Supplementary Figure S3C, http://links.lww.com/CM9/A38].

In this study, we reported the analysis of a cohort of patients with PCP and demonstrated that PD-$L1^+$ neutrophils may play a critical role in this disease. Although the role of neutrophils has previously been described in patients with PCP,[2,6] herein we report a significant correlation between high PD-L1-expressing neutrophils, $CD4^+$ T-cell suppression, and disease severity

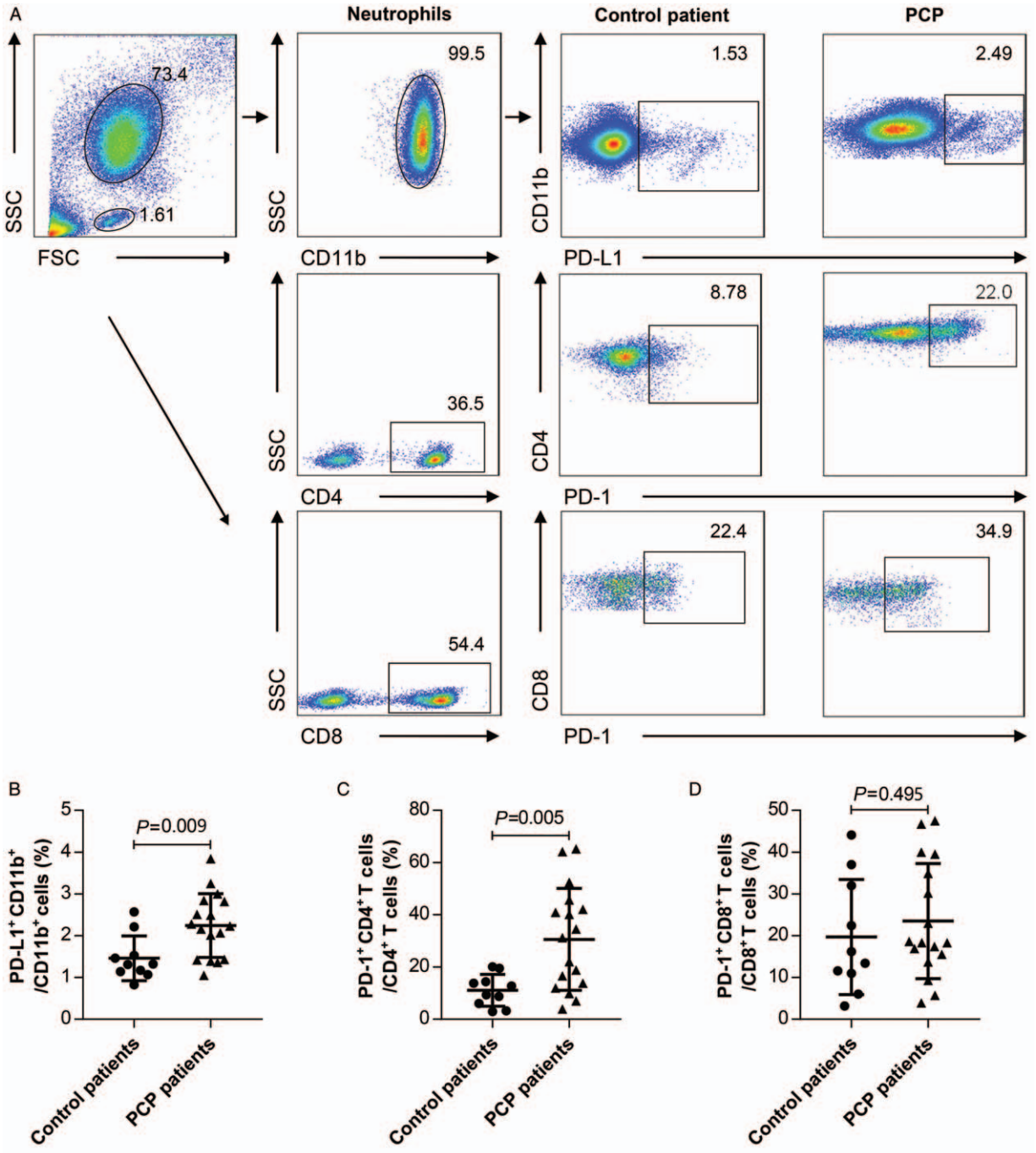

Figure 1: Expression of PD-L1 on circulating neutrophils is augmented in PCP patients. (A) Representative plot of PD-L1 expression on neutrophils and PD-1 expression on $CD4^+/CD8^+$ T cells derived from the blood of PCP and control patients. Frequency of PD-$L1^+$ neutrophils (B), PD-$1^+/CD4^+$ T cells (C), and PD-$1^+/CD8^+$ T cells (D) in the blood of patients with PCP ($n = 17$) and controls ($n = 10$). *P* values were calculated by Mann-Whitney test. FSC: Forward scatter characteristics; PCP: *Pneumocystis jirovecii* pneumonia; PD-L1: Programmed death ligand 1; SSC: Side scatter characteristics.

in patients. Moreover, the number of activated neutrophils, which are the predominant subset with high PD-L1 expression, correlated with the overexpression of PD-1 on $CD4^+$ T cells. These novel findings regarding PD-$L1^+$ neutrophils further our understanding of *Pneumocystis* pathogenesis and the connection between innate and adaptive immunity.

As reported, an elevated neutrophil count in BAL fluid can be used as a predictor of poor prognosis in HIV-negative patients with PCP.[2] This study significantly expands on these previous reports. We found that circulating neutrophils expressed higher levels of PD-L1 and that $CD4^+$ T cells overexpressed PD-1 in patients with PCP. Thus, we speculate that the role of neutrophils is likely to modulate the immunosuppressive function of T cells in patients with PCP. Augmented PD-1 expression on T cells derived from BAL fluid was recently demonstrated in one PCP case.[7] Therefore, additional investigation of the lungs is warranted.

The role of neutrophils in PCP immunopathogenesis is complex and controversial. Much of the controversy comes from the finding that simple elimination of neutrophils did not improve lung damage in a mouse model of the disease.[8] Furthermore, the incidence of *Pneumocystis* infection is rare in cases of immunodeficiency associated with neutropenia.[9] In contrast, in animal studies, neutrophil influx coincides with the presence of lung damage.[10] In the present study, we found a positive correlation between PD-L1$^+$ neutrophils and PD-1$^+$/CD4$^+$ T cells, indicating that neutrophils may suppress T cell immunity via activation of the PD-L1/PD-1 pathway. This finding adds a novel layer of complexity to our understanding of the role of neutrophils in PCP. We further highlighted a critical correlation between PD-L1$^+$ neutrophils, not only with PD-1$^+$/CD4$^+$ T cells, but also with disease severity. Therefore, our findings suggest a significant clinical role for PD-L1$^+$ neutrophils in PCP. However, we did not store neutrophils derived from blood during our study due to their short lifespan. Thus, the mechanism by which PD-L1-expressing neutrophils regulate T cell function in patients with PCP requires further investigation.

Neutrophils are a heterogeneous population. A recent report identified a subset of circulating CD16$^+$/CD62L^{low} neutrophils that were able to suppress T cell function in humans.[5] Our results indicate that PD-L1 phenotypic alterations were not present in the neutrophil population as a whole, but were limited to specific subsets, with increased PD-L1 expression observed on CD16$^+$CD62L^{low} subsets. Moreover, the percentage of activated PD-L1$^+$ neutrophils was positively correlated with the percentage of PD-1$^+$/CD4$^+$ T cells. Thus, we speculate that all PD-L1$^+$ neutrophils mediate CD4$^+$ T-cell suppression, particularly the activated subset.

While this work was performed with small sample sizes and the patient selection was limited to those admitted to the ICU, our results nonetheless show an association between PD-L1$^+$ neutrophils and PD-L1$^+$/CD4$^+$T cells, thus elucidating the connection between innate and adaptive immunity in patients with PCP. These findings improve our understanding of the role of neutrophils and provide a novel target for PCP immunotherapies.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patients have given their consent for their images and other clinical information to be reported in the article. The patients understand that their names and initials will not be published and due efforts will be made to conceal the identity of the patient, although anonymity cannot be guaranteed.

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Conflicts of interest

None.

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