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Lower expression of PD-1 and PD-L1 in peripheral blood from patients with chronic ITP

Jun Zhong1†, Shaohua Chen2†, Ling Xu2, Jing Lai1, Ziwei Liao2, Tao Zhang1, Zhi Yu1, Yuhong Lu1, Lijian Yang2, Xiuli Wu2, Bo Li2, Yangqiu Li1,2,3

1 Department of Hematology, First Affiliated Hospital, Jinan University, Guangzhou 510632, China, 2 Institute of Hematology, Medical College, Jinan University, Guangzhou 510632, China, 3 Key Laboratory for Regenerative Medicine of Ministry of Education, Jinan University, Guangzhou 510632, China

Background: T-cell dysregulation is a major event involved in immune thrombocytopenic purpura (ITP). Increasing data have indicated that abnormal expression of T-cell immunosuppressive receptors, such as programmed death (PD) 1 and cytotoxic T lymphocyte antigen-4 (CTLA-4), may be related to autoimmune disease pathogenesis.

Methods: We analyzed the expression levels of PD-1, its ligand PD-L1, and CTLA-4 in peripheral blood mononuclear cells from 18 patients with chronic ITP by real-time polymerase chain reaction, and samples from 20 healthy individuals served as control.

Results: The results demonstrated significantly lower expression of PD-1 (median: 0.0015) and PD-L1 (median: 0.0572) in chronic ITP patients compared with healthy individuals (PD-1: median: 0.0117, \( P < 0.0001 \); PD-L1: median: 0.5428, \( P < 0.0001 \)), while there was no significant difference in the CTLA-4 expression level between the chronic ITP patients (median: 0.0818) and healthy individuals (median: 0.1667) (\( P = 0.219 \)). Moreover, a positive correlation between the expression levels of PD-1 and PD-L1 (\( r_s = 0.486, P = 0.041 \)) and CTLA-4 and PD-1 (\( r_s = 0.643, P = 0.004 \)) in the chronic ITP patients was found.

Conclusion: Consistently lower expression of T-cell immunosuppressive receptors is a common characteristic of chronic ITP, which may be associated with its pathogenesis.

Keywords: Immune thrombocytopenic purpura, PD-1, PD-L1, CTLA-4

Introduction

Immune thrombocytopenic purpura (ITP) is an autoimmune disorder characterized by the production of auto-antibodies directed against platelet antigens. Increasing data have demonstrated that cytotoxic T cells, abnormal regulatory T cells (Tregs), helper T-cell imbalance, megakaryocyte maturation abnormalities, and abnormal T-cell anergy are involved in the pathogenesis of this disease, and alterations in the peripheral T-cell receptor (TCR) repertoire pattern and clonal expansion of TCR subfamily T cells have been characterized in ITP. While T-cell dysregulation plays a major role in this disease, alterations in the Treg frequency and functional characteristics might be responsible for the loss of self-tolerance and subsequent destructive immune responses that result in platelet destruction in ITP. Therefore, most studies of the immunopathogenic mechanisms associated with ITP have focused on how incompetent Tregs allow autoimmune effector mechanisms to proceed and cause thrombocytopenia, and there are some studies of T-helper 17 cells, related cytokines, and genetics. However, T-cell activation and dysfunction relies on direct and modulated receptors. Based on the functional outcome of TCR, their co-signaling molecules may be classified as co-stimulators or co-inhibitors, which positively and negatively control the priming, growth, differentiation, and functional maturation of T-cell responses. Increasing data have shown that loss of T-cell-mediated immune tolerance to platelet auto-antigens plays a crucial role such as decreased indoleamine 2,3-dioxygenase expression in dendritic cells in immune thrombocytopenia. Moreover, a new inducible co-stimulator signal transduction pathway, including the so-called T-cell immunosuppressive receptor programmed death (PD) 1 and cytotoxic T lymphocyte antigen-4 (CTLA-4), which are involved in the induction of T-cell tolerance,
plays an important role in immune homeostasis. Overexpression of these factors is related to tumor immunosuppression and poor prognosis, while down-regulation of these factors may be associated with an autoimmune disorder.\textsuperscript{15–19} Therefore, investigation of the characteristics of these factors can help improve our understanding of immune status and provide a new theoretical basis for studying the pathogenesis and treatment of ITP.

CTLA-4 is a CD28 surface marker homolog that plays an important role in the negative regulation of T-cell responses. Transient expression of this protein on the surface of activated T cells antagonizes activating signals and terminates T-cell responses.\textsuperscript{20,21} Recently, several studies have confirmed that some CTLA-4 polymorphisms may alter the antigen expression level and hence may influence immune regulation, thus suggesting that they may be associated with autoimmune diseases, such as autoimmune hemolytic anemia (AIHA). For example, the CTLA-4 exon 1 49 A>G polymorphism has recently been associated with AIHA, and CTLA-4 mutations were also detected in autosomal dominant immune dysregulation syndrome in humans,\textsuperscript{22} whereas no difference was observed in ITP patients and controls.\textsuperscript{20} Similar findings have shown that this polymorphism is not associated with susceptibility to ITP in the Egyptian population,\textsuperscript{23} and another report demonstrated that the –318 and CT60 polymorphisms in the CTLA-4 gene are not associated with susceptibility to ITP for 186 patients in the Chinese population.\textsuperscript{24}

PD-1 and programmed death 1 ligand 1 (PD-L1) are co-signaling molecules, and significant PD-1 and PD-L1 upregulation is related to immunosuppression in cancer,\textsuperscript{19,25,26} as well as enhancement the resistance to immunotherapy in cancer,\textsuperscript{27} while the major role of the PD-1 pathway is to inhibit self-reactive T cells and protect against autoimmune diseases. However, there are few reports regarding alterations in PD-1 and PD-L1 in ITP. Decreased serum-soluble PD 1 (sPD-1) and serum-soluble PD-L1 (sPD-L1) levels have been detected in ITP, and there was a positive correlation between sPD-1 levels and platelet counts.\textsuperscript{28} Therefore, it appears that alternative PD-1 and PD-L1 levels may play a role in ITP pathogenesis. In this study, we characterized the expression of PD-1, its ligand PD-L1, and CTLA-4 in peripheral blood mononuclear cells (PBMCs) from patients with chronic ITP.

Materials and methods

Samples

The study comprised 18 patients including 6 males and 12 females (median age: 38.5 years, range: 24–68 years) with chronic ITP. ITP diagnoses were based on the criteria reported in the guidelines of the American Society of Hematology.\textsuperscript{29} The clinical data of the patients are listed in Table 1. The baseline platelet count for all patients was under $100 \times 10^9/l$. Peripheral blood samples collected from 20 healthy donors with informed consent were used as control. All of the procedures were conducted according to the guidelines of the Medical Ethics Committees of the Health Bureau of the Guangdong Province of China, and ethical approval was obtained from the Ethics Committee of Medical School of Jinan University.

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<th>WBC count ($\times 10^9$/l)</th>
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<th>Gene expression</th>
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*Median.†Mean $\pm$ SD.
Quantitative real-time reverse transcription–polymerase chain reaction (qRT–PCR)

Mononuclear cells were isolated from peripheral blood (PBMCs) by Ficoll-Hypaque gradient centrifugation. RNA isolation and cDNA synthesis were performed according to the manufacturer’s protocol. The expression level of PD-1, PD-L1, CTLA-4, and the β2-microglobulin (β2-MG) reference gene was determined by SYBR Green I real-time PCR as previously described. The primers (Table 2) were purchased from Shanghai Sangon Biological Engineering Technology & Services Co. in China. PCR was performed as described in previous studies. Briefly, 25-μl PCRs were performed with approximately 1 μl cDNA, 0.5 μM of each primer (PD-1-for and PD-1-rev for the PD-1 gene, PD-L1-for and PD-L1-rev for the PD-L1 gene, CTLA-4-for and CTLA-4-rev for the CTLA-4 gene, and β2M-for and β2M-rev for the β2-MG gene), 2.5× RealMasterMix (11.25 μl) (Tiangen, Beijing). After an initial denaturation at 95°C for 2 hours, 45 cycles consisting of 95°C for 15 minutes and 60°C for 1 hour were performed using an MJ Research DNA Engine Opticon 2 PCR cycler (BIO-RAD, USA). The relative mRNA expression level of the detected genes in each sample was calculated using the comparative cycle time (Ct) method.

Statistical analyses

Differences in mRNA expression between two groups were analyzed using the Mann–Whitney test. Data are presented as medians. Spearman’s rank correlation analysis was used to analyze the PD-1, PD-L1, and CTLA-4 mRNA levels in different samples. Differences were considered statistically significant with a P < 0.05.

Results and discussion

T-cell dysregulation is a common characteristic of ITP pathogenesis. Increasing data have demonstrated abnormal T-cell immunity using different approaches, including T-cell proliferation, T-cell subsets, cytokine secretion, and T-cell activation. Moreover, numerous co-inhibitors and ligands, i.e. so-called T-cell immnosuppressive receptors or T-cell inhibitory receptors such as PD-1, PD-L1, and CTLA-4, which are expressed on T cells and dendritic cells, mediate T-cell immune suppression in cancer, and upregulation of these receptors is related to poor cancer prognosis. Blockade of PD-1, PD-L1, and CTLA-4 by antibodies was used in clinical trials for treatment of solid tumors such as lung cancer. In contrast, the major role of such T-cell immnosuppressive receptors is to inhibit self-reactive T cells and protect against autoimmune diseases. Low expression of such receptors may enhance the activation of T cells and be related to autoimmune disease pathogenesis. Few studies have examined alternative PD-1 and PD-L1 expression in ITP. The first study of the PD-1 signaling pathway in ITP was reported by Atesoglu et al. who

Table 2  List of primers used for real-time PCR

<table>
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<th>Primer</th>
<th>Sequence</th>
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<tr>
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<td>PD-1-rev</td>
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<td>CTLA-4-for</td>
<td>5′-AGACCTGAACAGCGCTCC-3′</td>
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<td>CTLA-4-rev</td>
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<tr>
<td>β2M-for</td>
<td>5′-TACGCTGAATGACCGCAGACG-3′</td>
</tr>
<tr>
<td>β2M-rev</td>
<td>5′-CATCCAATCAAATGCGGCA-3′</td>
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</table>

Figure 1 The expression levels of PD-1, PD-L1, and CTLA-4 in ITP patients and healthy individuals (HIs). Significantly decreased expression of PD-1 (A) and PD-L1 (B) was found, while the CTLA-4 expression level in the ITP group (C) appeared to be similar to that of the control group. The highest expression of PD-1, PD-L1, and CTLA-4 was found in the same ITP sample (case 5) (red points), and the lowest expression for all three genes was also found in the same ITP sample (case 10) (blue points).
found decreased levels of sPD-1 in 67 patients with ITP (24 with newly diagnosed ITP and 43 with chronic ITP).

In this study, we analyzed the expression of PD-1, PD-L1, and CTLA-4 in PBMCs from 18 patients with chronic ITP at the molecular level and compared these results with that of 20 healthy individuals. Significantly lower PD-1 (median: 0.0015) and PD-L1 (median: 0.0572) expression was found in chronic ITP compared with healthy samples (PD-1, median: 0.0117, \( P < 0.0001 \); PD-L1, median: 0.5428, \( P < 0.0001 \)) (Fig. 1A and B). Lower expression of PD-1 and PD-L1 may indicate the status with reduced T-cell immunosuppression which may result in abnormal T-cell activation in chronic ITP patients, however, further investigation is needed in larger cohort of samples to confirm the results. The finding also supported the results from Atesoglu et al.\(^{28}\) However, this group found that the sPD-L1 level was decreased in patients with newly diagnosed ITP compared with those with chronic ITP, and there was no significant difference compared with healthy individuals.\(^{28}\) The finding seemed differently from the present study, we found that the PD-L1 expression level was also significantly decreased in chronic ITP in comparison with healthy control, the reason may be due to different analysis target, the expression level of PD-L1 gene represents the total PD-L1 mRNA from PBMCs, while the level of sPD-L1 represents the part of PD-L1 which is soluble in serum rather than the total of PD-L1 level. Moreover, we found a positive correlation between the PD-1 and PD-L1 expression levels in ITP (\( r_s = 0.486, P = 0.041 \)) (Fig. 2A). Interestingly, there was no significant correlation between the expression level of both genes in healthy individuals (\( r_s = 0.044, P = 0.846 \)). These results may indicate that the general PD-1 and PD-L1 status in healthy individuals was altered during the abnormal immune status activation in the chronic ITP patients. The changes in the PD-1 and PD-L1 levels may be linked to the platelet count. Atesoglu et al.\(^{28}\) described a positive correlation between serum sPD-1 levels and

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**Figure 2** The correlation between the expression levels of PD-1, PD-L1, and CTLA-4 in ITP patients and healthy individuals (HIs). (A) PD-1 vs. PD-L1 in ITP; (B) PD-1 vs. PD-L1 in HI; (C) PD-1 vs. CTLA-4 in ITP; (D) PD-1 vs. CTLA-4 in HI; (E) PD-L1 vs. CTLA-4 in ITP; (F) PD-L1 vs. CTLA-4 in HI.
platelet counts. In this study, we found no significant correlation between $PD-I$ ($r_s = 0.422, P = 0.081$) or $PD-L1$ ($r_s = 0.260, P = 0.297$) and platelet counts; thus, it may be difficult to explain the direct association between these T-cell immunosuppressive receptors. Studies involving a larger ITP sample set are needed to help clarify the role of these receptors. Overall, our findings suggest that PD-1 and PD-L1 may play a role in chronic ITP pathogenesis. Moreover, it will be interesting to further characterize the expression pattern of different factors involved in different T-cell subsets.

Before the identification and application of PD-1 and PD-L1, most studies have focused on the association between CTLA-4 and autoimmune disorders and included not only changes in expression level but also the characterization of mutations and polymorphisms in this gene in ITP. However, the significance of CTLA-4 variants was different in different studies. For example, a study of the −318 and CT60 polymorphisms in the $CTLA-4$ gene, which were confirmed to influence the $CTLA-4$ expression level in 186 patients with ITP, indicated that these two single-nucleotide polymorphisms are not associated with susceptibility to ITP in a Chinese population. Similarly, the $CTLA-4$ exon 1 49 A>G polymorphism is not associated with susceptibility to ITP in the Egyptian population, and it does not affect the clinical presentation of this disease. In this study, we also examined the $CTLA-4$ expression level in the chronic ITP samples, and there was no significant difference in $CTLA-4$ expression when comparing the chronic ITP (median: 0.0818) and healthy groups (median: 0.1667) ($P = 0.219$) (Fig. 1C). However, we found a positive correlation between the $CTLA-4$ and $PD-1$ expression levels ($r_s = 0.643, P = 0.004$), but there was no significant correlation between $CTLA-4$ and $PD-L1$ ($r_s = 0.222, P = 0.576$). As shown in Fig. 1, the expression levels of $PD-I$, $PD-L1$, and particularly $CTLA-4$ were relatively different in the chronic ITP samples. Interestingly, the highest expression levels for $PD-I$, $PD-L1$, and $CTLA-4$ were found in the same chronic ITP sample (case 5), and the lowest expression level for these three genes was also demonstrated in the same chronic ITP sample (case 10). Such an identical gene expression pattern was not previously observed. These findings suggest that the immune suppression status in chronic ITP may be consistent for the three factors, and these may be as additional abnormal immune biomarkers for chronic ITP, however, further investigation is needed to characterize the relationship with other T-cell immunosuppressive receptors, e.g. Tim-3 and LAG-3, to describe a global view of the T-cell immune suppression status in ITP and different autoimmune disorders. Moreover, it would be worthy of investigating the role of such T-cell immunosuppressive receptors in stem cell memory T cells, a new subtype of memory T cells in ITP.

**Disclaimer statements**

**Contributors** Y.Q.L. contributed to the concept development and study design. J.Z., S.H.C., L.X., Z.W.L., L.J.Y., X.L.W., and B.L. performed the experiments and analyzed the data. J.Z., J.L., T.Z., Z.Y., and Y.H.L. were responsible for clinical diagnoses and performed clinical data acquisition. Y.Q.L., J.Z., and S.H.C. coordinated the study and helped draft the manuscript. All authors read and approved the final manuscript.

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**Conflicts of interest** The authors declare that they have no competing interests.

**Ethics approval** All of the procedures were conducted according to the guidelines of the Medical Ethics Committees of the Health Bureau of the Guangdong Province of China, and ethical approval was obtained from the Ethics Committee of Medical School of Jinan University.

**References**

26 Schnorfel FM, Lichtenenegger FS, Emmerig K, Schlueer M, Neitz JS, Draenert R, et al. T cells are functionally not impaired in AML: increased PD-1 expression is only seen at time of relapse and correlates with a shift towards the memory T cell compartment. J Hematol Oncol. 2015;8:93.