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ORIGINAL ARTICLE

The abnormal function of CD39$^+$ regulatory T cells could be corrected by high-dose dexamethasone in patients with primary immune thrombocytopenia

Yumeng Lu[1,2] · Luya Cheng[1] · Feng Li[1,3] · Lili Ji[1] · Xia Shao[1] · Boting Wu[4] · Yanxia Zhan[1] · Chanjuan Liu[1] · Zhihui Min[5,6] · Yang Ke[1] · Lihua Sun[3] · Hao Chen[7,8] · Yunfeng Cheng[1,3,5,6]

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Abstract

Primary immune thrombocytopenia is an autoimmune disease, characterized with decreased platelet and increased risk of bleeding. Recent studies have shown the reduction and dysfunction of regulatory T (Treg) cells in ITP patients. CD39 is highly expressed on the surface of Treg cells. It degrades ATP to AMP and CD73 dephosphorylates AMP into adenosine. Then adenosine binds with adenosine receptor and suppresses immune response by activating Treg cells and inhibiting the release of inflammatory cytokines from effector T (Teff) cells. Adenosine receptor has several subtypes and adenosine A2A receptor (A2AR) plays a crucial role especially within lymphocytes. The CD39$^+$ Treg cells and the expression of A2AR showed abnormality in some autoimmune disease. But knowledge of CD39$^+$ Treg cells and A2AR which are crucial in the adenosine immunosuppressive pathway is still limited in ITP. Thirty-one adult patients with newly diagnosed ITP were enrolled in this study. CD39 and A2AR expression was measured by flow cytometry and RT-PCR. The function of CD39 was reflected by the change of ATP concentration detected by CellTiter-Glo Luminescent Cell Viability Assay. CD39 expression within CD4$^+$CD25$^+$ Treg cells in ITP patients was decreased compared to normal controls. After high-dose dexamethasone therapy, response (R) group showed increased CD39 expression within Treg cells while non-response (NR) group did not show any difference in contrast to those before treatment. The expression of A2AR in CD4$^+$CD25$^-$ Teff and CD4$^+$CD25$^+$ Treg cells was both lower in ITP patients than that of normal controls. After therapy, CD4$^+$CD25$^-$ Teff cells had higher A2AR expression while CD4$^+$CD25$^+$ Treg cells did not show any difference in comparison to that before treatment. The enzymatic activity of CD39 was damaged in ITP patients and improved after high-dose dexamethasone therapy. In ITP, there was not only numerical decrease but also impaired enzymatic activity in CD39$^+$ Treg cells. After high-dose dexamethasone treatment, these two defects could be reversed.

Yumeng Lu and Luya Cheng contributed equally to this work.

✉ Hao Chen
h.chen@fudan.edu.cn

✉ Yunfeng Cheng
yfcheng@fudan.edu.cn

Yumeng Lu
08301010268@fudan.edu.cn

Luya Cheng
cheng.luya@zs-hospital.sh.cn

Feng Li
li.feng@zs-hospital.sh.cn

Lili Ji
ji.lili@zs-hospital.sh.cn

Xia Shao
16111210011@fudan.edu.cn

Boting Wu
wu.boting@zs-hospital.sh.cn

Yanxia Zhan
zhan.yanxia@zs-hospital.sh.cn

Chanjuan Liu
15111210015@fudan.edu.cn

Zhihui Min
min.zhihui@zs-hospital.sh.cn

Yang Ke
ke.yang@zs-hospital.sh.cn

Lihua Sun
qpsunlh023@126.com

Extended author information available on the last page of the article

Our results also suggested that ITP patients had reduced A2AR expression in both $CD4^+CD25^+$ Treg cells and $CD4^+CD25^-$ Teff cells. $CD4^+CD25^-$ Teff cells had increased A2AR expression after treatment.

Keywords Immune thrombocytopenia · Regulatory T cell · CD39 · Adenosine receptor · A2AR · Dexamethasone

Background

Primary immune thrombocytopenia is an autoimmune disease, characterized with decreased platelet and increased risk of bleeding [1, 2]. Besides platelet-specific antibodies produced by auto-reactive B cells [3] and imbalance of Th1/Th2 cell [4–6], study of regulatory T (Treg) cell abnormalities is getting more and more attention. Lots of studies have revealed both the numerical and functional defect of Treg cells in ITP [7–17]. Effective treatment can significantly increase Treg cell amount and restore their function [7–11, 14–17]. Usually, Treg cells were measured by the expression of Foxp3 in $CD4^+CD25^+$ cells; Foxp3 transcription factor can be used as a specific molecular marker for Treg cells. However, its location inside the cell limits its application in some Treg cell studies. Researches have shown that CD39 is highly expressed on the surface of Treg cells and has been increasingly used as a marker of Treg cells [18, 19]. In mouse, CD39 is expressed in almost all Foxp3+ regulatory T cells. In human, CD39 is mainly expressed in regulatory T cells with memory activation. CD39 could be a marker which is independent of Foxp3, and CD39 subgroup could also be considered a subtype of Treg cells [18, 19]. CD39 is an integral membrane protein and a member of ecto-nucleoside triphosphate diphosphohydrolase family (E-NTPDase) [20]. Of this family, CD39 is the dominant ectoenzyme in the immune system [21, 22]. CD39 degrades extracellular adenosine triphosphate (ATP) to adenosine monophosphate (AMP), and CD73 (ecto-5′-nucleotidase, Ecto5'NTase) dephosphorylates AMP into adenosine [23, 24]. This conversion is considered one of the mechanisms by which Treg cells reduce inflammation. Then adenosine binds with adenosine receptor on the membrane and suppresses immune response by activating Treg cells and inhibiting the release of inflammatory cytokines from Teff cells [25]. Treg cells separated from CD39 knockout mice lost their function to suppress the proliferation of Teff cells [18]. It shows that CD39 plays a crucial part in immunoregulation. Adenosine receptor has several subtypes: A1R/A2AR/A2BR/A3R and A2AR play a crucial role especially within lymphocytes [26–29]. Treg and Teff cells both express A2AR on their cellular membrane. Therefore, the expression of CD39 and the enzymatic activity of CD39 determine the production of adenosine. And the expression of adenosine A2A receptor determines the effect of adenosine immunosuppressive pathway.

Defective numbers of $CD39^+$ Treg cells have been reported in multiple sclerosis, where these cells were also impaired in their ability to suppress IL-17 production [30–32]. The abnormal amount and dysfunction of $CD39^+$ Treg cells have been found in other autoimmune diseases such as autoimmune hepatitis, rheumatoid arthritis, inflammatory bowel disease, and systematic lupus erythematosus [33–37]. The expression of adenosine A2A receptor also showed abnormality in myasthenia gravis and type 2 diabetes mellitus [38, 39] which were considered to be immune-involved diseases. Given the key role of CD39 and A2AR in the adenosine immunosuppressive pathway, our current study aimed to explore $CD39^+$ Treg cell amount, the expression of A2AR in both Treg and Teff cells, and the enzymatic activity of CD39 within Treg cells.

Subjects and methods

Patients and controls

Thirty-one adult patients with newly diagnosed ITP according to an international working group [1, 40, 41] were enrolled in the study (Table 1). Their blood platelet count was less than 30×10^9/L and therefore they required medical intervention. Pregnant females, secondary ITP, and those who could not undergo corticosteroid treatment due to certain reasons like contraindications were excluded. All patients received HD-DXM regimen (40 mg of oral dexamethasone daily for 4 consecutive days). Before and one month after treatment, 10 ml venous blood samples of patients and normal controls was collected. Patients whose platelet counts were remarkably increased and efficacy was validated in accordance with the Vicenza Consensus Conference were described as the response (R) group [1, 40, 41]. Patients whose platelet counts were not remarkably increased were described as the non-

Table 1 Clinical characteristics of ITP patients

Characteristics	All patients ($N = 31$)	
	No.	%
Gender		
Female	22	71
Male	9	29
Age, years median (range)	48 (22, 72)	–
Platelet counts ($\times 10^9$/L), median (range)		
Before treatment	15 (1, 29)	–
After treatment	122 (10, 358)	–

response (NR) group [1, 40, 41]. Patients were paired with their own before (Pre-T group) and after treatment (Post-T group). Healthy volunteers were taken as normal controls (NC group).

The study was approved by local Medical Ethics Committees of Zhongshan Hospital, Fudan University. Written informed consent was obtained from each patient enrolled in the study.

Sample preparation

Venous blood samples were collected in ethylenediaminetetraacetic acid–treated tubes and diluted 1:2 with Hanks balanced salt solution (HBSS) before Ficoll-Hypaque gradient centrifugation (2500 rpm at room temperature for 15 min). Washed and resuspended, isolated peripheral blood mononuclear cells (PBMCs) were cryopreserved in fetal bovine serum containing 10% dimethyl sulfoxide (DMSO), and stored in liquid nitrogen for future flow cytometric analysis (FCM) and real-time polymerase chain reaction (RT-PCR).

Cell culture

Cryopreserved PBMCs were thawed at 37 °C, washed twice with HBSS, and stained with trypan blue to testify cell viability. PBMCs were seeded at 5×10^5/mL in 24-well plates in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 200 U/mL penicillin, and 100 μg/mL streptomycin for 24 h.

Flow cytometric analysis

Cultured cells were stained with FITC-conjugated anti-CD4/APC-conjugated anti-CD25/PE-conjugated anti-CD39 antibodies at 4 °C for 20 min, washed twice, and resuspended in staining buffer for analysis according to the manufacturer's protocol (ebioscience, USA). For adenosine A2A receptor intracellular-staining, the cells were incubated with FITC-conjugated anti-CD4/APC-conjugated anti-CD25 monoclonal antibodies and then fixed with fixative buffer for 20 min. Afterward, the cells were permeabilized with permeabilization buffer and stained with PE-conjugated anti-A2A monoclonal antibodies (Santa Cruz Biotechnology, Inc.) for 30 min in the dark. Stained cells were tested on a FACS Aria II flow cytometer (BD, USA) and then analyzed using Flowjo software version 7.6.

Real-time polymerase chain reaction

Total RNA was isolated from PBMCs by Trizol reagent (Invitrogen, USA) and converted into cDNA using a Prime-Script RT reagent kit (Takara, Japan) according to the manufacturer's instructions. CD39 and A2AR gene expression was quantified using the SYBR Premix Ex Taq (Takara, Japan) on a MasterCycler Realplex4 system (Eppendorf, German), with GAPDH expression as a control. Amplification was performed in a total volume of 20 μL for 40 cycles of 5 s at 95 °C and 30 s at 60 °C after initial denaturation (95 °C, 30 s). The primer sequences were as follows: GAPDH-sense: 5′-CATC AGCAATGCCTCCTGCAC-3′; GAPDH-antisense: 5′-TGAGTCCTTCCACGATACCAAAGTT-3′; CD39-sense: 5′-CCATCCTTGGCTTCTCCTCTAT-3′; CD39-antisense: 5′-CCACGCCTGTGTCATTCTCCT-3′; A2AR-sense: 5′-CACGAGGGCTAAGGGCATCATT-3′; A2AR-antisense: 5′-GCAGTCGGGGCAGAAGAAAGT-3′. Samples were analyzed in triplicate. 2-ΔΔCt was used to calculate fold change of mRNA expression.

ATP hydrolysis assay

PBMCs of the same density were incubated in medium containing 10 μM ATP for 30 min. ATP concentration in the supernatant was determined using the CellTiter-Glo Luminescent Cell Viability Assay (Promega cat. no. G7570). ATP standard luminescence curve was drawn using the ATP disodium salt (Sigma cat. no. A7699).

Statistical analysis

All analyses were performed with SPSS 19.0 software. Continuous variables data were expressed as mean ± SD. Normality was assessed by Shapiro-Wilk test and homogeneity of variances was evaluated by Levene's test. One-way ANOVA and Kruskal-Wallis test were used for data that fulfilled normal distribution and for those that did not, respectively. Paired student *t* test was used to evaluate the differences between patients before and after therapy. A value of $p < 0.05$ was considered statistically significant.

Results

The percentage of $CD4^+CD25^+$ Treg cells was decreased in ITP patients and could be corrected by effective corticosteroid therapy

Groups were age and sex matched. The result showed a notable decrease of the percentage of $CD4^+CD25^+$ Treg cells in the Pre-T group compared with the NC group (NC vs. Pre-T—(10.22 ± 7.77)% vs. (3.80 ± 3.54)%, $p < 0.001$, Fig. 1b). Among the response group (R), the Post-T group possessed significantly higher $CD4^+CD25^+$ Treg cells percentage than the Pre-T group (Pre-T vs. Post-T—(4.59 ± 3.99)% vs. (8.52 ± 5.02)%, $p = 0.011$, Fig. 1c) and no difference between the

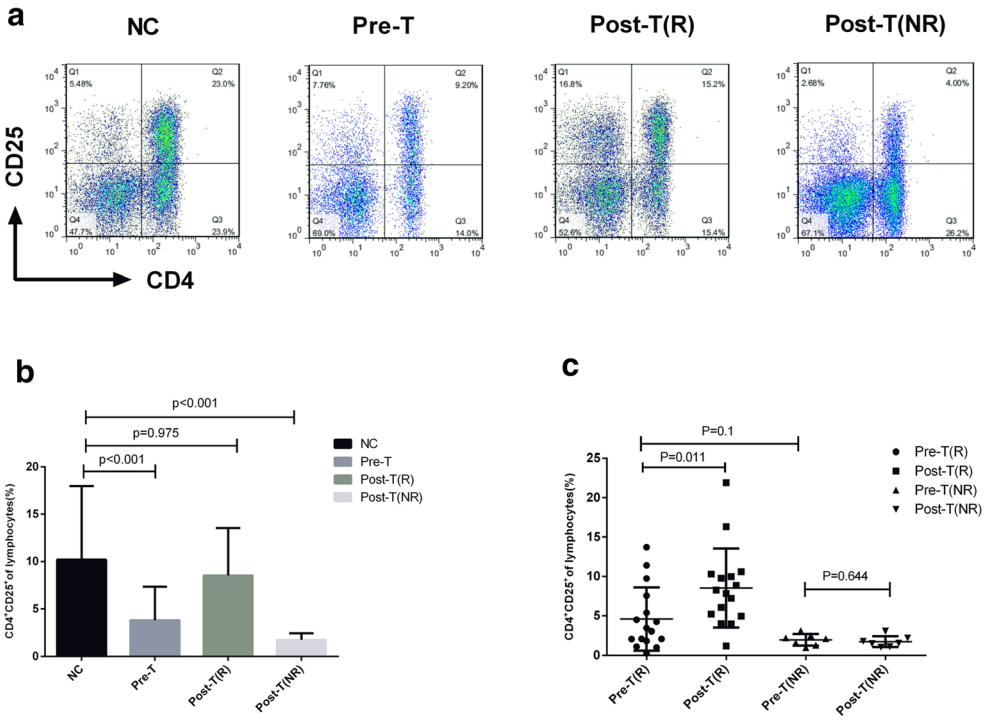

Fig. 1 Frequency of $CD4^+CD25^+$ cells was decreased in ITP patients and increased after treatment in the response group. **a** The representative dot plots of $CD4^+CD25^+$ cells within lymphocytes in different groups. **b**, **c** The mean ± SD of percentage of $CD4^+CD25^+$ cells within lymphocytes in different groups. *p* value is shown in the figure. NC, normal control ($n = 20$). Pre-T, ITP patients before treatment ($n = 23$). Post-T, ITP patients after treatment ($n = 23$). R, response group ($n = 16$). NR, non-response group ($n = 7$)

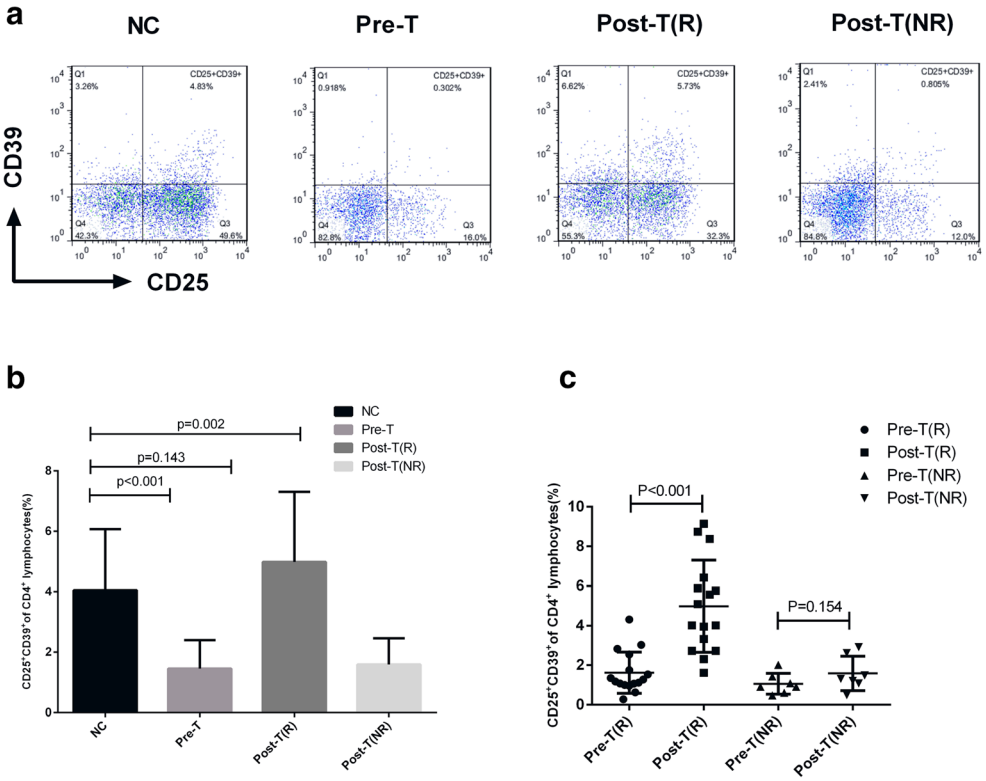

Fig. 2 $CD25^+CD39^+$ cells within $CD4^+$ lymphocytes was reduced in ITP patients and elevated after treatment in the response group. **a** The representative dot plots of $CD25^+CD39^+$ cells within $CD4^+$ lymphocytes in different groups. **b**, **c** The mean ± SD of percentage of $CD25^+CD39^+$ cells within $CD4^+$ lymphocytes in different groups. *p* value is shown in the figure. NC, normal control ($n = 20$). Pre-T, ITP patients before treatment ($n = 23$). Post-T, ITP patients after treatment ($n = 23$). R, response group ($n = 16$). NR, non-response group ($n = 7$)

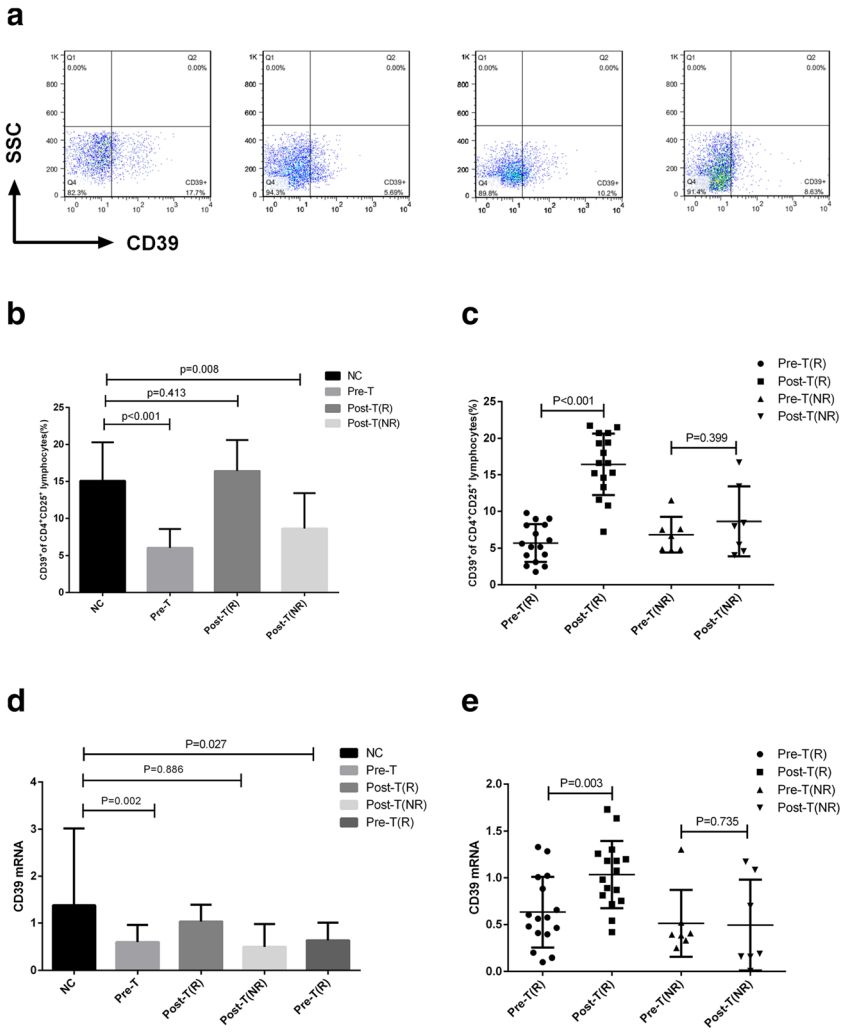

Fig. 3 Expression of CD39 in Treg cells was decreased in ITP patients and increased after treatment in the response group. **a** The representative dot plots of $CD39^+$ cells within Treg cells in different groups. **b**, **c** The mean ± SD of percentage of $CD39^+$ cells within Treg cells in different groups. *p* value is shown in the figure. **d** The mean ± SD of CD39 mRNA within lymphocytes in different groups. **e** The mean ± SD of CD39 mRNA within lymphocytes in ITP patients before and after treatment. *p* value is shown in the figure. NC, normal control ($n = 20$). Pre-T, ITP patients before treatment ($n = 23$). Post-T, ITP patients after treatment ($n = 23$). R, response group ($n = 16$). NR, non-response group ($n = 7$)

NC group and the Post-T group ($p = 0.975$, Fig. 1b). Among the non-response group (R), there is no difference between the Pre-T group and the Post-T group (Pre-T vs. Post-T—(1.07 ± 0.53)% vs. (1.59 ± 0.87)%, $p = 0.644$, Fig. 1c). The representative dot plots are shown in Fig. 1a.

The expression of CD39 in $CD4^+CD25^+$ Treg cells was decreased in ITP patients and could be corrected by high-dose dexamethasone therapy

Groups were age and sex matched. A population of $CD25^+CD39^+$ cells within $CD4^+$ lymphocytes and $CD39^+$ cells within $CD4^+CD25^+$ lymphocytes were detected, which were both remarkably lower in the Pre-T group than in the NC group ($p < 0.001$, $p < 0.001$, respectively; Fig. 2b, Fig. 3b). The CD39 mRNA of PBMCs was also decreased in the Pre-T group compared with the NC group (NC vs. Pre-T—1.38 ± 1.64 vs. 0.59 ± 0.37, $p = 0.002$, Fig. 3d). Among the response group (R), both the population of $CD25^+CD39^+$ cells within $CD4^+$ lymphocytes and $CD39^+$ cells within $CD4^+CD25^+$ lymphocytes were increased compared with the Pre-T group ($p < 0.001$, $p < 0.001$, respectively; Fig. 2c, Fig. 3c). On the other hand, among the non-response group (NR), there was no difference between the Pre-T and Post-T groups ($p = 0.154$, $p = 0.399$, respectively, Fig. 2c, Fig. 3c). The representative dot plots are shown in Fig. 2a and Fig. 3a.

In accordance with CD39 staining, the expression of CD39 mRNA in PBMCs was also elevated after therapy at the R group (Pre-T vs Post-T—0.63 ± 0.38 vs. 1.03 ± 0.36, $p = 0.003$, Fig. 3e) and showed no difference at the NR group (Pre-T vs Post-T—0.51 ± 0.36 vs. 0.50 ± 0.49, $p = 0.735$, Fig. 3e).

The expression of adenosine A2A receptor in $CD4^+$ lymphocytes was decreased in ITP patients

Groups were age and sex matched. The expression of A2A receptor in both $CD4^+CD25^-$ Teff cells and

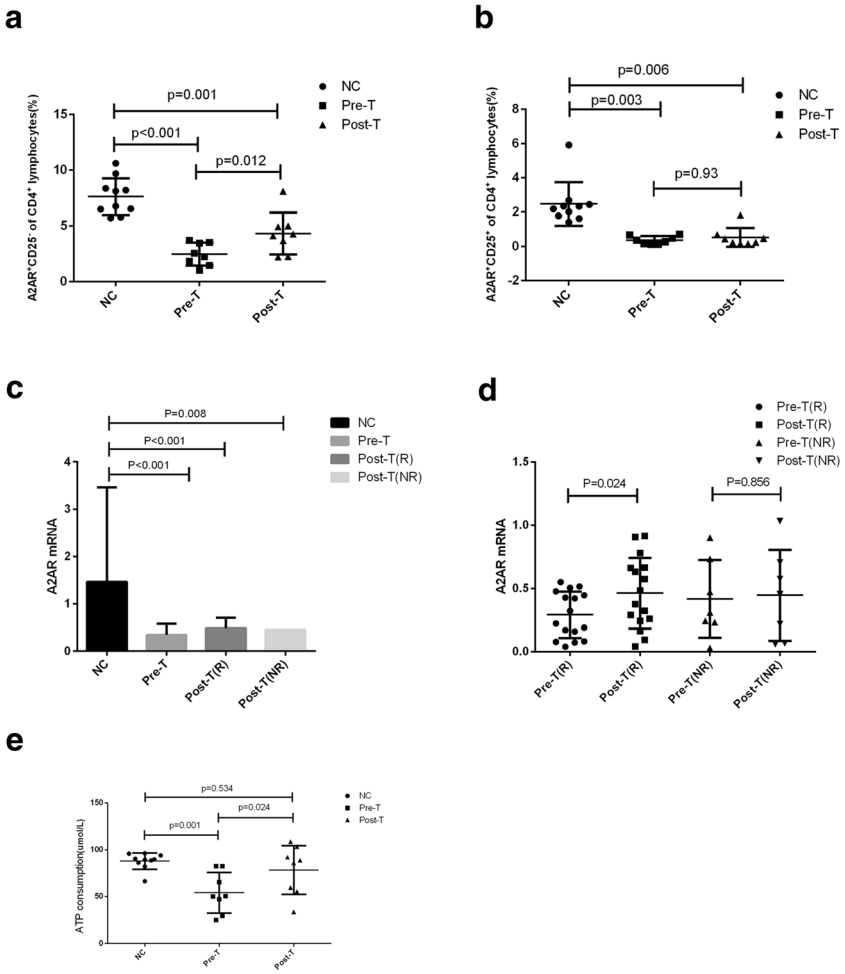

Fig. 4 Expression of adenosine A2A receptor and enzymatic activity of CD39 was decreased in ITP patients. **a** The mean ± SD of percentage of $A2AR^+$ $CD25^-$ cells within $CD4^+$ lymphocytes in different groups. **b** The mean ± SD of percentage of $A2AR^+$ $CD25^+$ cells within $CD4^+$ lymphocytes in different groups. *p* value is shown in the figure. NC, normal control ($n = 10$). Pre-T, ITP patients before treatment ($n = 8$). Post-T, ITP patients after treatment ($n = 8$). **c** The mean ± SD of A2AR mRNA within lymphocytes in different groups. **d** The mean ± SD of A2AR mRNA within lymphocytes in ITP patients before and after treatment. *p* value is shown in the figure. A2AR, adenosine A2A receptor. NC, normal control ($n = 20$). Pre-T, ITP patients before treatment ($n = 23$). Post-T, ITP patients after treatment ($n = 23$). R, response group ($n = 16$). NR, non-response group ($n = 7$). **e** The mean ± SD of ATP consumption in different groups. *p* value is shown in the figure. NC, normal control ($n = 10$). Pre-T, ITP patients before treatment ($n = 8$). Post-T, ITP patients after treatment ($n = 8$)

$CD4^+CD25^+$ Treg cells has been assessed. The percentage of $A2A^+$ $CD25^-$ cells within $CD4^+$ lymphocytes in both Pre-T group and Post-T groups was decreased compared with that in the NC group ($p < 0.001$, $p = 0.001$, respectively, Fig. 4a). The same result went for $A2A^+$ $CD25^+$ cells within $CD4^+$ lymphocytes ($p = 0.003$, $p = 0.006$, respectively, Fig. 4b). The frequency of $A2A^+$ $CD25^-$ Teff cells was elevated in the Post-T group (Pre-T vs Post-T—(2.47 ± 1.02)% vs. (4.31 ± 1.86)%, $p = 0.012$, Fig. 4a), whereas no significant difference was observed between the Pre-T group and the Post-T group in $A2A^+$ $CD25^+$ cells (Pre-T vs Post-T—(0.36 ± 0.24)% vs. (0.53 ± 0.55)%, $p = 0.93$, Fig. 4b). The A2AR mRNA of PBMCs was also decreased in both Pre-T group and Post-T groups compared with that in the NC group ($p < 0.001$ (Pre-T), $p < 0.001$ (Post-T R), $p = 0.008$ (Post-T NR), respectively, Fig. 4c). A2AR mRNA in PBMCs was elevated after therapy at the R group ($p = 0.024$, Fig. 4s) and showed no difference at the NR group ($p = 0.856$, Fig. 4D).

The enzymatic activity of CD39 was damaged in ITP patients and could be improved after therapy

Groups were age and sex matched. To test for the functional activity of CD39, the consumption of exogenous ATP by PBMCs was assessed. Ectonucleotidase activity was lower in the Pre-T group than in the NC group (NC vs. Pre-T—87.87 ± 8.70 vs. 54.09 ± 21.51, $p = 0.001$, Fig. 4e). But it is increased after therapy (Pre-T vs. Post-T—54.09 ± 21.51 vs. 78.42 ± 26.05, $p = 0.024$, Fig. 4e).

Discussion

Previous investigations have found CD39 defects of $CD4^+CD25^+$ (we called Treg cells in this study) cells in several immune-related diseases, such as autoimmune hepatitis and multiple sclerosis [30–32, 34]. Inconsistent with these studies, we found that the expression of CD39 in $CD4^+CD25^+$Treg cells was decreased at both protein and mRNA levels in ITP patients compared with normal controls. Apart from that, our research also revealed that $CD39^+$ Treg cells were impaired in the ability to hydrolyze ATP and ADP in ITP patients in comparison to normal controls. Those two defects led to a reduced production of immunosuppressive adenosine and persistently high levels of pro-inflammatory ATP in ITP patients which lead to activation and perpetuation of inflammation. After high-dose dexamethasone treatment, the amount and the hydrolytic activity of $CD39^+$ Treg cells were both restored. However, among patients who had no response to corticosteroids, CD39 expression remained impaired in $CD4^+CD25^+$ Treg cells. These results suggested that expression and function of CD39 in $CD4^+CD25^+$ Treg cells may be correlated with the state of disease and may contribute to the initiation and perpetuation of ITP. CD39 may also be involved in therapeutic effect and low expression can possibly result in lack of response to treatment. Improvement of CD39 expression and function may be involved in the remission and recovery after effective treatment. Furthermore, CD39 may be used to assess the therapeutic effect of ITP and potential therapy for boosting CD39 expression in ITP should be explored in the future. Although CD25 is widely used to identify Treg cells, its specificity is limited because its expression is also induced upon activity of conventional T cells. CD39 could help in the characterization of Treg cells and is likely to be used as a novel functional marker. Expression of CD39 is regulated mainly by two transcriptional factors: sp1 and stat3 which can be triggered by hypoxia, ischemia, and oxidative stress [42, 43]. Moreover, low expression of CD39 in inflammatory bowel disease was reported to be associated with CD39 polymorphism, suggesting a genetically encoded defect of immunoregulation in this condition [33]. Exploration of upstream signals such as sp1 and factors such as CD39 polymorphism which may account for the defect of CD39 in patients with ITP deserves extended studies.

Among $CD4^+$ T lymphocytes, the activation of adenosine A2A receptor can suppress the production of IL-2, IL-4, and IFN-γ; upregulate the expression of CTLA-4 and PD-1; and downregulate the expression of CD40L [44–47]. A2AR stimulation in Treg cells can also increase the expression of Foxp3 known as a transcription factor and a maker of Treg cells [48, 49]. Researches have found the abnormality of adenosine A2A receptor expression in some immune-related diseases, such as myasthenia gravis and type 2 diabetes mellitus [38, 39]. In our study, we demonstrated that the expression of A2A adenosine receptor in both Treg cells and Teff cells was diminished in ITP patients in contrast to normal controls. After effective corticosteroid treatment, Teff cells expressed more A2AR but no changes were observed in Treg cells. These findings indicated that the low expression of A2AR in $CD4^+$ T lymphocytes could play a crucial role in the immune disorder in ITP. In experimental autoimmune myasthenia gravis (EMAG), Na Li et al. revealed the administration of A2AR agonist CGS21680 not only led to a decrease in anti-AChR IgG levels but also partially restored the imbalance between Th1/Th2/Th17/Treg cell subset. Furthermore, preventive treatment of EAMG with CGS21680 was effective in down-modulating disease manifestations and therapeutic treatment partly attenuated the severity of established EAMG [38]. Among other autoimmune diseases such as inflammatory bowel disease and experimental autoimmune encephalomyelitis, A2A receptor agonists also showed effective in animal models [50–52]. Moreover, A2A receptor agonists, through activation of Tregs and immune tolerance, have proven to be effective in reducing and preventing graft versus host disease (GVHD) development in murine models of the disease [53, 54]. On the opposite side, improving anti-tumor T cell activity by A2AR antagonists suggested promise for disengaging the adenosine-mediated immunosuppression in the tumor microenvironment like non-small-cell lung cancer [55, 56]. Therefore, ongoing development of therapeutic strategies targeting the A2AR may have beneficial therapeutic applications in ameliorating severity of disease and offer promise for the management of some autoimmune diseases such as ITP.

In conclusion, this study has shown that, in ITP, there was a numerical decrease in CD39 expression of $CD4^+CD25^+$ cells. These $CD39^+$ Treg cells were impaired in their enzymatic and suppressive abilities. After effective corticosteroid treatment, these two defects could be reversed. Our results also demonstrated that ITP patients had reduced A2AR expression in both $CD4^+CD25^+$ Treg cells and $CD4^+CD25^-$ Teff cells. Therefore, reduced production of adenosine and decreased expression of adenosine A2A receptor in $CD4^+$ T lymphocytes may be both involved in the development of ITP. Therapies targeting adenosine production and A2AR such as A2AR agonists may have a promising future.

Author contribution YL and YC conceived the study; YL, LC, FL, LJ, and YC performed the literature review, and drafted and revised the manuscript; YL and YC contributed to the critical revision of the manuscript; YL, YZ, LC, XS, BW, CL, ZM, YK, LS, and HC performed the experiments and analyzed the data. All authors read and approved the final manuscript.

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Compliance with ethical standards

The study was approved by local Medical Ethics Committees of Zhongshan Hospital, Fudan University. Written informed consent was obtained from each patient enrolled in the study.

Competing interests The authors declare that they have no conflict of interest.

Data sharing statement Data sharing: no additional data available

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Affiliations

Yumeng Lu[1,2] · Luya Cheng[1] · Feng Li[1,3] · Lili Ji[1] · Xia Shao[1] · Boting Wu[4] · Yanxia Zhan[1] · Chanjuan Liu[1] · Zhihui Min[5,6] · Yang Ke[1] · Lihua Sun[3] · Hao Chen[7,8] · Yunfeng Cheng[1,3,5,6]

1 Department of Hematology, Zhongshan Hospital Fudan University, Shanghai 200032, China

2 Department of Hematology, Shanghai Tenth People's Hospital, Tongji University, Shanghai 200072, China

3 Department of Hematology, Zhongshan Hospital Qingpu Branch, Fudan Universiy, Shanghai 201700, China

4 Department of Transfusion Medicine, Zhongshan Hospital Fudan University, Shanghai 200032, China

5 Institute of Clinical Science, Zhongshan Hospital, Fudan University, Shanghai 200032, China

6 Shanghai Institute of Clinical Bioinformatics, Fudan University Center for Clinical Bioinformatics, Shanghai 200032, China

7 Department of Thoracic Surgery, Zhongshan Hospital Xuhui Branch, Fudan University, Shanghai 200031, China

8 Institute of Clinical Science, Department of Hematology, Zhongshan Hospital, Fudan University, 180 Fenglin Rd, Shanghai 200032, China