Int J Clin Exp Pathol 2015;8(12):15642-15651
www.ijcep.com /ISSN:1936-2625/IJCEP0018134

Original Article

Expression of CD11a in lymphocyte subpopulation in immune thrombocytopenia

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Received October 19, 2015; Accepted November 28, 2015; Epub December 1, 2015; Published December 15, 2015

Abstract: Recent research demonstrates that the underlying mechanism in immune thrombocytopenia (ITP) is very complex. Lymphocyte function associated antigen-1 (LFA-1) plays important roles in autoimmune diseases. The purpose of this study was to investigate the expression of CD11a on lymphocytes and explore its possible role in ITP. The expression of CD11a on lymphocyte subpopulations ($CD3^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD4^-$ T cells, $CD4^+Foxp3^+$ T regulatory cells and $CD19^+$ B cells) were analyzed by flow cytometry. Specific anti-platelet GPIIb/IIIa and/or GPIb/IX autoantibodies were assayed by modified monoclonal antibody specific immobilization of platelet antigens (MAIPA). The mean fluorescence intensity of CD11a on $CD3^+$ T, $CD3^+CD4^-$ T and $CD19^+$ B lymphocytes were increased in ITP patients compared to healthy controls. No significant difference of CD11a expression on $CD3^+CD4^+$ T cells or $CD4^+Foxp3^+$ T regulatory cells was found between ITP patients and controls. Our data indicates the possible role of CD11a in the pathogenesis of ITP.

Keywords: Immune thrombocytopenia, LFA-1, CD11a, B cells, T cells

Introduction

Immune thrombocytopenia (ITP) is an autoimmune disease characterized by low platelet counts with or without mucocutaneous bleeding [1]. Like the majority of autoimmune diseases, ITP is an organ-specific disease and abnormalities in the regulation of immune system have been shown to play an important role in the initiation and/or perpetuation of the disease [2]. Autoantibodies reacting against platelet glycoproteins can mediate platelet destruction by the monocyte-macrophage system as well as suppress megakaryocyte proliferation and maturation [3]. Although autoreactive B lymphocytes secreting antiplatelet antibodies are considered as the main defect, substantial evidence suggests that a generalized dysfunction of autoreactive T cells is the critical immunopathological cause of ITP and the antiplatelet autoantibodies are under the control of T cells and the cytokines they produce [4]. In the current management of ITP, corticosteroids and intravenous immunoglobulin are the first-line choice, and many drugs including rituximab have also been tried as second-line treatments. However, the curative effect is unsatisfactory.

Lymphocyte function associated antigen-1 (LFA-1) belonging to the integrin family is composed of the alpha chain CD11a and beta chain CD18 heterologous dimers [5], and expressed on the surface of T lymphocytes, B lymphocytes, monocytes, macrophages and neutrophils. Its major ligand, intercellular adhesion molecule-1 (ICAM-1), belongs to the immunoglobulin superfamily, distributed on the surface of fibroblasts, endothelial cells and activated lymphocytes. The combination of LFA-1 and ICAM-1 can provide coordinated stimulus signal and promote lymphocyte activation, proliferation and differentiation. In the interaction of T cells with antigen-presenting cells (APCs), LFA-1 and its adaptor ICAM-1 directly participate in the formation of immunological synapse that promotes costimulatory function, leading to increased T cell proliferation and cytotoxicity [6]. They also take part in a variety of lymphocytic homing process. In addition, the interaction of LFA-1 with ICAM-1 can also mediate a series of inflammatory responses [7]. CD11a is critical for lymphocyte entry into the lymph nodes [8] and normal development of hematopoietic intermediates [9]. The disruption of LFA-1 activity strongly affects the stability of immune interface [10].

Table 1. Clinical characteristics of ITP patients

Patients	Gender	Age (year)	PLT count ($\times 10^9$)	Anti-GPIIb/IIIa anti-GPIb/IX	Bleeding symptoms
1	Male	46	15	-/+	PT, EC
2	Male	52	25	-/-	EC, GUH
3	Male	25	40	+/-	EC
4	Female	57	10	-/-	PT, EC
5	Female	24	2	+/-	PT, GH
6	Male	39	1	-/-	EC, GU
7	Male	46	4	-/+	EC, GUH
8	Male	42	16	+/+	PT
9	Male	19	55	-/-	EC
10	Female	66	14	-/-	PT, EC
11	Female	49	48	+/-	PT
12	Male	41	33	+/+	EC
13	Female	75	2	-/+	EC, GU
14	Female	61	5	-/-	PT, GH
15	Female	53	6	+/-	GH
16	Female	58	10	-/+	PT, EC
17	Fale	36	35	-/-	EC
18	Female	38	25	-/-	GU
19	Female	26	23	-/-	PT, EC
20	Female	39	9	+/+	PT, GH
21	Female	51	14	-/-	GH
22	Male	40	22	-/+	GUH
23	Female	52	50	-/-	EC
24	Male	43	3	-/-	EP, GU
25	Male	24	0	+/+	EC, EP, GU
26	Male	46	7	-/+	EC, EP
27	Female	39	2	-/-	EP, GU
28	Male	42	23	-/-	EC
29	Female	78	91	-/+	PT
30	Male	62	3	-/+	EC, EP, GU
31	Female	58	13	-/-	EP, GU
32	Male	46	63	-/-	PT
33	Female	25	61	+/-	EC
34	Female	32	51	-/-	GU

*PT = petechiae, EC = ecchymoses, EP = epistaxis, GH = gingival hemorrhage, GUH = genitourinary hemorrhage.

Therefore, LFA-1 plays important roles in inflammatory, immune responses and antigen presentation.

The expression of ICAM-1 and LFA-1 is significantly higher on lymphoid cells and vascular endothelial cells in rheumatoid arthritis (RA), indicating that the combination of LFA-1 and ICAM-1 may play an important role in the progression of RA [11]. The excessive expression of LFA-1 can induce the formation of auto-reactive T cells, resulting in lupus disease in mice. By using LFA-1 monoclonal antibodies in lupus mice the production of autoantibodies could be reduced, the development of autoimmune reaction stopped, and the symptoms of lupus nephritis alleviated [12]. Therefore, LFA-1 may play an important role in the pathogenesis of systemic lupus erythematosus. It has been reported that CD11a was elevated in ITP patients and CD11a could facilitate the survival of $CD19^+$ B cells and promote antibody-mediated platelets destruction [13]. In this study we analyzed the CD11a expression on $CD4^+$ $Foxp3^+$ T cells, $CD3^+CD4^+$ T cells, $CD3^+CD4^-$ T cells and $CD19^+$ B cells, respectively, and compared its expression between ITP patients and healthy controls.

Materials and methods

Patients

Thirty-four patients (eighteen females and sixteen males with a median age of 45 years, **Table 1**) admitted to our wards were studied. Patients with diabetes, heart disease, pregnancy or any other autoimmune disease were excluded. The diagnosis of ITP was based on the recently reported criteria [14]. Nineteen healthy controls with matched sex and age were also studied. Enrollment took place between February 2013 and January 2014 at the Department of Hematology of our hospital. Informed consent was obtained from each participant. Ethical approval for the study was obtained from the Medical Ethical Committee.

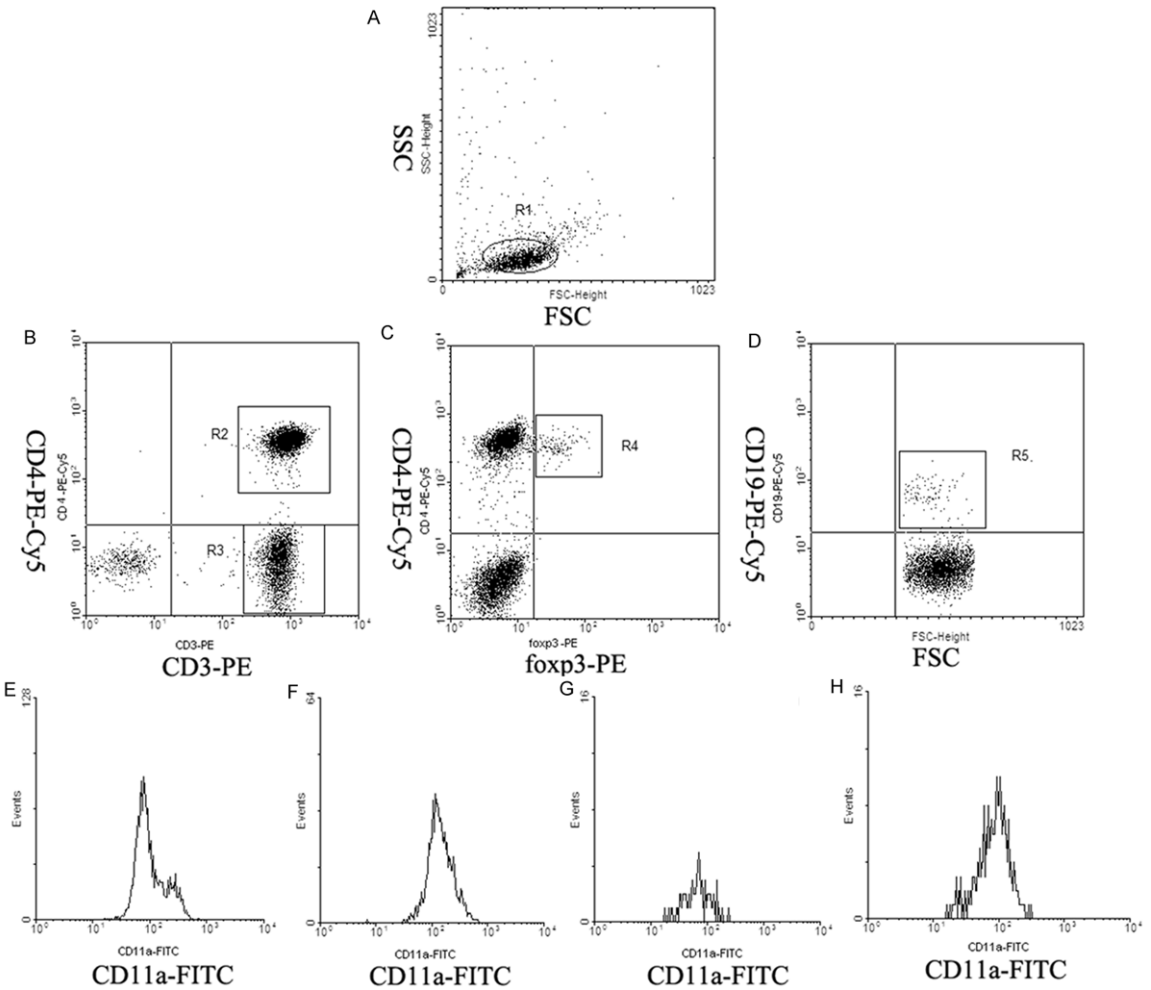

Figure 1. The expression of CD11a on lymphocyte subsets. (A) Lymphocytes were gated by flow cytometry (R1). (B) $CD3^+CD4^+$ T cells (R2) and $CD3^+CD4^-$ T cells (R3) were gated by flow cytometry. (C) $CD4^+foxp3^+$ Treg cells (R4) were gated by flow cytometry. (D) $CD19^+$ B cells (R5) were gated by flow cytometry. (E-H) showed histogram of the expression of CD11a gated on R2 (E), R3 (F), R4 (G) and R5 (H).

Isolation of peripheral blood mononuclear cells (PBMCs)

Eathylene diamine tetraacetic acid (EDTA) anticoagulant venous blood was collected from ITP patients and healthy controls. PBMCs were isolated using Ficoll-Hypaque (Invitrogen, Carlsbad, CA) density gradient Centrifugation at 2,000 rpm for 20 min at 20°C and washed twice with phosphate-buffered saline (PBS).

Flow cytometric analysis

The expression of CD11a on lymphocyte subsets was analyzed by flow cytometry. PBMCs were adjusted to 5×10^6/ml, 100 μl (5×10^5 cells) were incubated at 4°C for 30 min with fluorescence antibodies. The following monoclonal antibodies (eBiosciences, San Diego, CA) were used for surface antigen staining: anti-CD11a monoclonal antibody conjugated to fluorescein isothiocyanate, anti-CD3 conjugated to phycoerythrin (PE), anti-CD4 conjugated to PE-Cy5 and anti-CD19 conjugated to PE-Cy5. Cells were washed with 2 ml PBS and centrifuged at 1500 rpm for 5 min. Cell pellets were then resuspended in 0.3 ml PBS and analyzed by flow cytometer (BD Biosciences, San Jose, CA).

To determine the expression of CD11a on T regulartory cells (Tregs), PE-conjugated Foxp3 antibody reagent kit and a fixation/permeabilization solution and permeabilization buffer (eBiosciences, San Diego, CA) were used for intracellular Foxp3 staining according to the manufacturer's instructions. After 100 μl PBMCs were incubated at 4°C for 30 min with

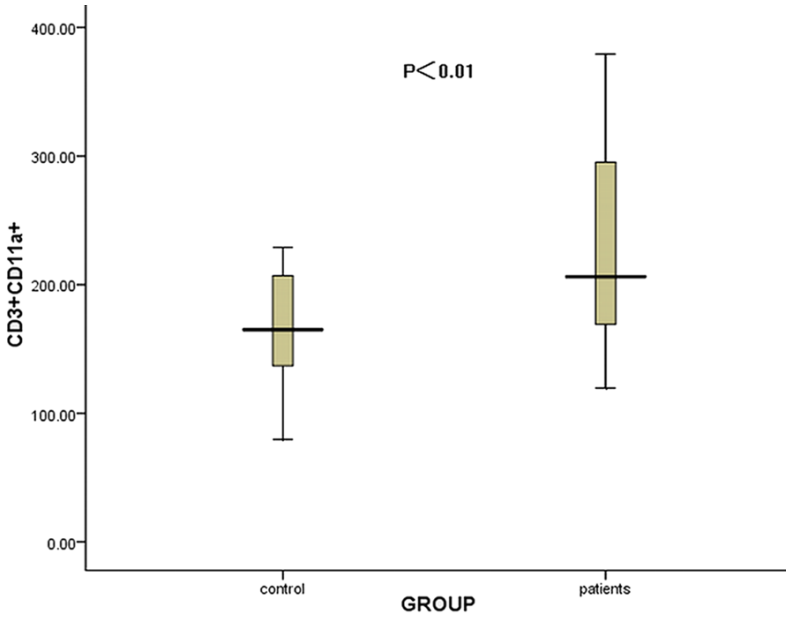

Figure 2. The comparison of the CD11a MFI on $CD3^+$ T cells between ITP patients and controls. The expression of CD11a on $CD3^+$ T cells was significantly increased in ITP patients compared to controls (P<0.01).

anti-CD11a monoclonal antibody conjugated to fluorescein isothiocyanate and anti-CD4 conjugated to PE-Cy5, 1 ml of 1 × Foxp3 Fix/Perm buffer solution was added to each tube. The cells were resuspended and incubated in the dark for 20 min, centrifuged and removed of the supernatants and washed twice. Then the cells were resuspended in 1 ml 1 × Foxp3 Perm buffer, incubated in the dark for 15 min. After centrifugation, the supernatant was discarded and the pellet resuspended in 100 μl of 1 × Foxp3 Perm buffer. Appropriate amount of flurochrome conjugated anti-Foxp3 antibody was added and incubated in the dark for 30 min. The cells were washed twice with staining buffer, resuspended in 0.5 ml cell staining buffer and analyzed with flow cytometer. Negative and isotype controls were used for each sample.

Modified monoclonal antibody specific immobilization of platelet antigens (MAIPA) assay

All plasma samples were stored at -20°C prior to use. The MAIPA assay of ITP patients with specific anti-platelet GPIIb/IIIa and/or GPIb autoantibodies was carried out as previously described in detail by Hou et al [15].

Statistical analysis

Data were expressed as mean ± standard deviation (SD). The difference between means was tested by independent-student *t* tests. The Pearson correlation test was conducted to identify univariate associations. All tests were performed by SPSS 16.0 system. Differences with a $P<0.05$ were determined as statistically significant.

Results

The expression of CD11a was analyzed by flow cytometry. **Figure 1** showed the expression of CD11a on lymphocyte subsets. $CD3^+CD4^+$ T cells (R2), $CD3^+CD4^-$ T cells (R3), $CD4^+foxp3^+$ Treg cells (R4) and $CD19^+$ B cells (R5) were gated by flow cytometry. **Figure 1E-H** showed histogram of the expression of CD11a gated on $CD3^+CD4^+$ T cells, $CD3^+CD4^-$ T cells, $CD4^+foxp3^+$ Treg cells and $CD19^+$ B cells.

Expression of CD11a on T cells

CD11a was expressed on all lymphocyte subsets in patients and healthy controls. The expression of CD11a mean fluorescence intensity(MFI) on total T cells ($CD3^+$) were increased in ITP patients compared to healthy controls (mean ± SD, 221.47 ± 68.33 & 165.57 ± 43.93, respectively, $P<0.01$) (**Figure 2**).

The CD11a MFI on $CD3^+CD4^-$ T lymphocytes were increased in ITP patients compared to healthy controls (259.97 ± 88.79 & 193.58 ± 46.30, respectively, $P<0.05$) (**Figure 3A**). While no significant difference of CD11a MFI on $CD3^+CD4^+$ T lymphocytes was found between patients and controls (160.69 ± 50.84 & 143.40 ± 21.69, respectively, $P>0.05$) (**Figure 3B**).

Expression of CD11a on Treg cells

The percentage of $CD4^+foxp3^+$ T cells in ITP patients was obviously lower than that in normal controls ($P<0.05$, data not shown). No significant difference of CD11a MFI on $CD4^+foxp3^+$ Treg cells was found between patients and controls (160.69 ± 50.84 & 143.40 ± 21.69, respectively, $P>0.05$) (**Figure 4**).

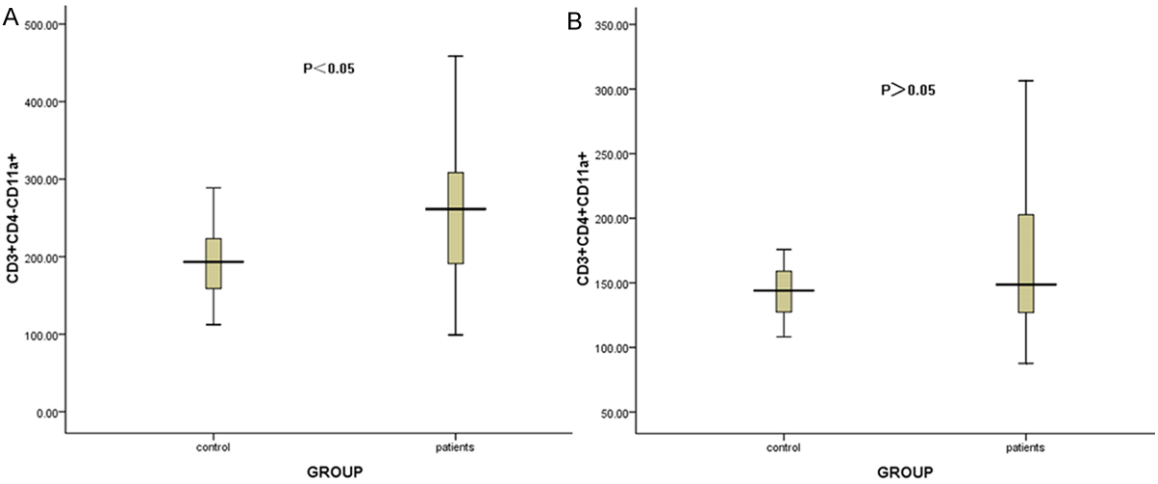

Figure 3. The comparison of the CD11a MFI on $CD3^+CD4^-$ and $CD3^+CD4^+$ T lymphocytes between ITP patients and healthy controls. A showed the comparison of the CD11a MFI on $CD3^+CD4^-$ T lymphocytes between ITP patients and healthy controls (P<0.05). B showed that CD11a expression on $CD3^+CD4^+$ T lymphocytes was not significantly different between patients and controls (P>0.05).

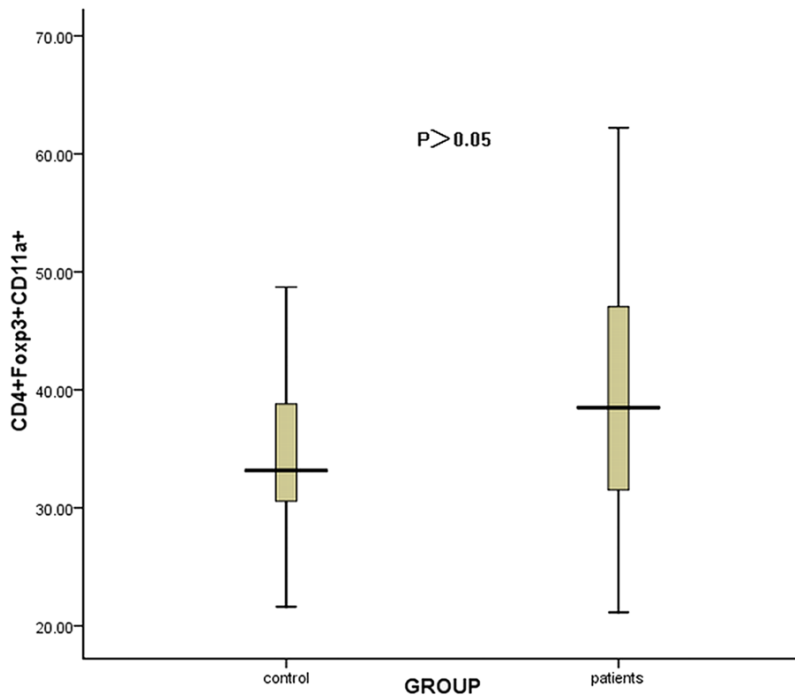

Figure 4. The comparison of the CD11a MFI on $CD4^+foxp3^+$ Treg cells between ITP patients and controls. No significant difference of the CD11a MFI on $CD4^+foxp3^+$ Treg cells was found between patients and controls (P>0.05).

Expression of CD11a on B cells

The percentage of $CD19^+$ B lymphocytes in peripheral blood of ITP patients was significantly increased compared with that in healthy controls (P<0.05, data not shown). The CD11a MFI on $CD19^+$ B lymphocytes was increased in ITP patients compared to controls (75.84 ± 27.61 & 56.26 ± 21.05, respectively, P<0.05) (**Figure 5**).

Correlation of CD11a expression between different lymphocyte subsets

The CD11a expression on $CD3^+CD4^-$ T cells was positively correlated with that on $CD19^+$ B cells (P<0.05, r = 0.365) (**Figure 6**), while no significant correlation of CD11a between other lymphocyte subsets was found (P>0.05).

Correlation between CD11a expression with platelet counts or anti-platelet autoantibodies

No significant correlation was found between CD11a expression on lymphocyte subsets with platelet counts or anti-platelet autoantibodies in ITP patients (P>0.05).

Discussion

ITP is an autoimmune disease characterized by low platelet count, increased bleeding tendency, blood smear showing a decreased number

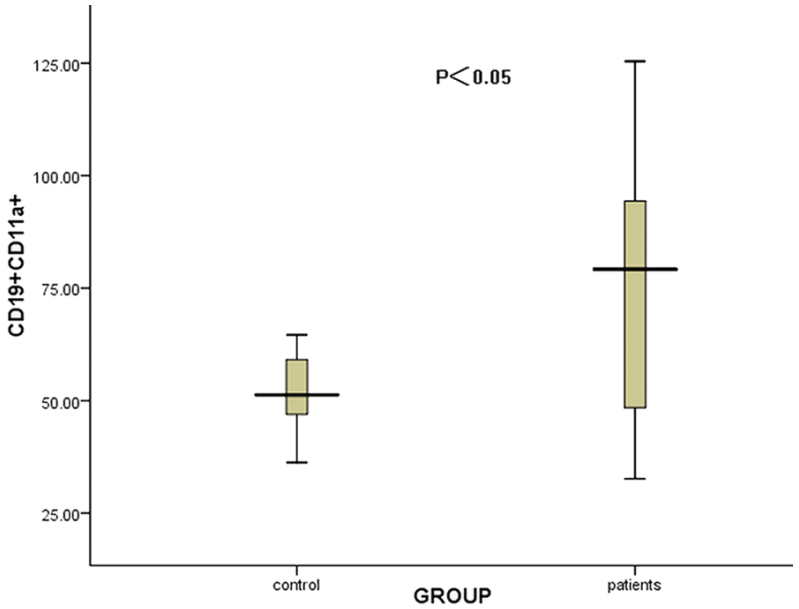

Figure 5. The comparison of the CD11a expression on $CD19^+$ B lymphocytes between ITP patients and controls. The CD11a expression on $CD19^+$ B lymphocytes was significantly increased in ITP patients compared to controls ($P<0.05$).

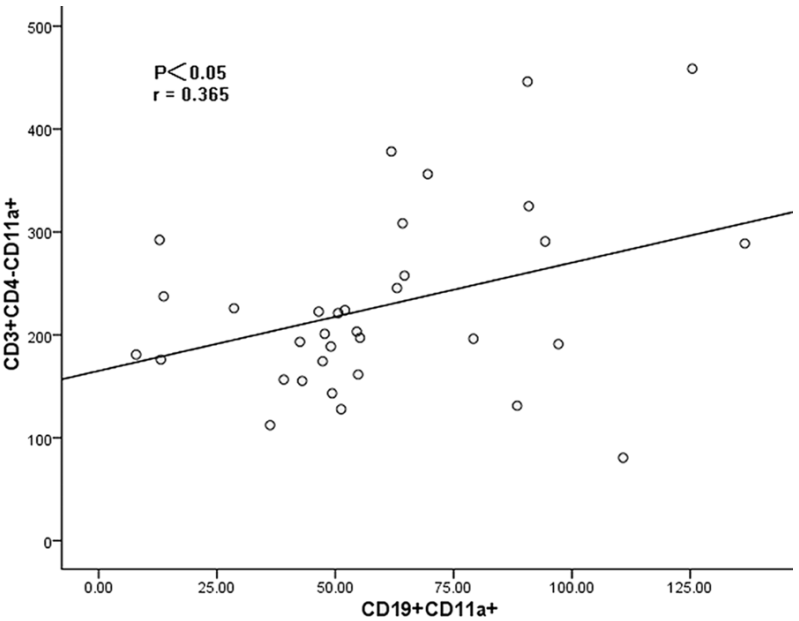

Figure 6. The correlation between the CD11a expression on $CD3^+CD4^-$ cells and that on $CD19^+$ cells. The CD11a expression on $CD3^+CD4^-$ cells was positively correlated with that on $CD19^+$ cells ($r = 0.365$, $P<0.05$).

of platelets, bone marrow test showing an increased number and immature of megakaryocytes. Autoantibodies against platelet surface glycoproteins such as GPIIb/IIIa and GPIb/IX complexes, and cytotoxic T cell mediated platelet destruction play important roles in its pathophysiology. Recent research demonstrates that the underlying mechanism in ITP is very complex and includes: decreased number and defective suppressive function of Tregs, Th1/Th2 imbalance, increase of Th17 cells and IL-17 levels, elevated Th22 cells, decreased B regulatory cells, and so on [16-21].

In the current study the expression of CD11a on all lymphocyte subsets were analyzed in ITP. We found the expression of CD11a on $CD3^+$ T cells, $CD3^+CD4^-$ T lymphocytes and $CD19^+$ B lymphocytes were increased in ITP patients compared to healthy controls. CD11a on $CD3^+CD4^+$ T lymphocytes and $CD4^+Foxp3^+$ Tregs were not significantly different between patients and controls.

During adaptive immunity, T cells become activated and proliferated in lymphoid organs when encountering antigens presented by APCs. After activation, T cells arrive at the site of inflammation and exert active roles in peripheral tissues [22]. T-cell recruitment, which is controlled by the combination of adhesion molecules and their receptors, is crucial for T-cell circulation among lymphoid organs and peripheral tissues [23]. Dysregulation of T cell activity and cytokine abnormalities have been found to be a contributing factor to many autoimmune diseases, including ITP [24]. LFA-1 is the predominant cell adhesion molecule present on T cells [25] and plays cru-

cial roles in lymphocyte trans-endothelial migration, immunologic synapse and T-cell activation and proliferation through interaction with ICAM-1 on APCs [26]. LFA-1 can decrease the threshold of TCR signals and antigen doses required for T cell activation and proliferation in vitro [25, 27]. Increasing CD11a expression in T cells by transfection causes autoreactivity in vitro and an autoimmune disease in mice [28]. In our study CD11a was expressed on all T cells and the expression of CD11a was increased on $CD3^+$ T cells in ITP patients compared with healthy controls, confirming LFA-1 plays a critical role on T cell activation and increased CD11a expression on T cells may induce autoreactivity in ITP.

T cells are broadly classified as either helper T cells (Th cells, $CD4^+$) or cytotoxic T cells (Tc cells, $CD8^+$). Th cells play a key role in determining whether autoantibodies are produced. They are separated into Th1 cells and Th2 cells, depending upon the specific cytokines which the cells secrete in response to antigenic stimulation [29]. There were some studies showing an increased Th1/Th2 ratio in ITP patients compared with controls [18]. In addition to their roles in adaptive immunity, memory T cells, $CD8^+$ T cells in particular, are also involved in innate immunity, becoming activated and proliferated under cytokine stimulation in the absence of antigens. In some research, it may be speculated that Tc cells play a part in at least some patients with ITP, Tc cells may directly lyse platelets and possibly suppress megakaryopoiesis. CD11a appears to play a direct role on effector CD8 T cell subset development, presumably through augmentation of T cell-APC interactions [30]. In the current study, the expression of CD11a on $CD3^+CD4^-$ T lymphocytes was increased in ITP patients compared to healthy controls and that on $CD3^+CD4^+$ T lymphocytes was not significantly different between patients and controls. We speculated that Th1 cells bias and Tc cells could both participate in the occurrence of ITP and Tc cells may lyse platelets partly through LFA-1-dependent pathway. However, $CD4^+$ T cells may play a part in LFA-1-independent manner.

Tregs are a subset of T cells, representing 5-10% of peripheral $CD4^+$ T cells. Marked by their expression of CD4, CD25, CD127 and Foxp3, they appear to play a critical role in maintaining peripheral tolerance by suppressing both cell-mediated and antibody-mediated responses [31]. Evidence indicated that levels of circulating Tregs in patients with acute and chronic ITP have been significantly reduced compared to healthy controls [32, 33]. In ITP, platelet autoreactive T cells have decreased apoptosis, underwent clonal expansion and created a cytokine imbalance that can lead to lower levels and abnormal function of Tregs [34]. Defective Tregs appear to be critical to the pathogenesis of ITP by breaking self-tolerance, allowing the autoimmune process to progress [17]. This shift in self-tolerance was believed to contribute to autoimmune-mediated platelet destruction. LFA-1 on the human Tregs is critical for their suppressor-function. Tran DQ found that Tregs could not exert its inhibition with blocking anti-human CD11a or anti-human CD18a monoclonal antibody, and Tregs in patients lack of LFA-1 could not restrain T cell activation [35, 36]. As the researcher points out, antigen-activated Tregs exert suppression by two distinct steps: initial LFA-1-dependent formation of Treg aggregates on immature dendritic cells (DCs) and subsequent LFA-1- and CTLA-4-dependent active down-modulation of CD80/86 expression on DCs. Both steps prevent antigen-reactive naive T cells from being activated by antigen-presenting DCs, resulting in specific immune suppression and tolerance [37]. In our study we detected the number of $CD4^+Foxp3^+$ Tregs and the expression of CD11a on Tregs, the results verified the decreased number of Tregs in ITP. However, the expression of CD11a in ITP was not significantly different compared with healthy controls. Reasons for this finding may include too little sample number, insensitive methods and so on. In addition, we speculated that Tregs may also exert its inhibition through other pathways in ITP.

The interaction of LFA-1 and its ligands provides essential adhesive and co-stimulatory signals required for the initiation of immune responses. B cells are generally considered to be positive regulators of the immune response because of their capability to produce antibodies, including autoantibodies. Autoantibodies against platelets really play a major role in ITP. Increased levels of circulating B cells excreting anti-GPIIbIIIa antibodies in ITP patients have been reported [38]. The dysregulation of B cell development is also associated with ITP. LFA-1/

ICAMs interaction transduces biochemical signals for T-cell-dependent B cell activation and immunoglobulin production and lowers the threshold of B cell activation by facilitating B cell adhesion and synapse formation [39]. Anti-LFA-1 added to culture strongly inhibits antibody responses although B-cell proliferative responses are only partially mitigated [40]. We speculated that CD11a over-expression on $CD19^+$ cells in ITP could provide sufficient B cell stimulatory signals to activate B cells and produce autoantibodies.

The positive correlation of CD11a expressions between $CD3^+CD4^-$ cells and $CD19^+$ cells indicated that Tc cells and B cells could play their roles through LFA-1 participated common pathway in ITP. In our study the CD11a expressions on all lymphocyte cells were not relevant with the platelet counts or anti-platelet autoantibodies in ITP, indicating that the expression levels of CD11a on lymphocyte cells can not reflect the severity of the disease.

Taken together, elevated expression of CD11a on $CD3^+CD4^-$ cells and $CD19^+$ cells indicate its possible role in the pathogenesis of ITP, even though further experimentation is needed to elucidate the basic mechanism by which LFA-1 exert its function.

Acknowledgements

This work was supported by grants from National Natural Science Foundation of China (81100335, 81202307 and 81100417).

Disclosure of conflict of interest

None.

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