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ORIGINAL ARTICLE

Neutrophils contribute to elevated BAFF levels to modulate adaptive immunity in patients with primary immune thrombocytopenia by CD62P and PSGL1 interaction

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Abstract

Objectives. Immune thrombocytopenia (ITP) is an autoimmune disease characterised by impaired platelet production and increased platelet destruction. However, the involvement of neutrophils in ITP is yet to be explored. **Methods.** B-cell activating factor (BAFF) expression and activation markers of neutrophils, as well as activation of platelets in ITP patients and healthy controls were measured. The interaction of CD62P on platelets and BAFF in neutrophils was analysed by correlation analysis and verified by co-culture. The effects of neutrophils on apoptosis of acquired immune cells were evaluated in co-culture systems with or without belimumab. **Results.** The BAFF expression and activation of neutrophils were increased in active ITP patients. BAFF levels in neutrophils were positively correlated with $CD62P^+$ platelets and neutrophils produced increased BAFF by interfering with CD62P on platelets. Neutrophils inhibited the apoptosis of $CD4^+$, $CD8^+$ and $CD19^+$ cells dependent on BAFF levels, and belimumab could interrupt the effects of neutrophils. **Conclusions.** Neutrophils were overactivated in ITP patients and participated in the progression of disease by producing excessive BAFF, which could be regulated by CD62P on platelets. Targeting BAFF by belimumab may be a novel potential therapy for ITP.

Keywords: acquired immunity, BAFF, CD62P, neutrophils, primary immune thrombocytopenia

INTRODUCTION

Primary immune thrombocytopenia (ITP) is a heterogeneous autoimmune disease characterised by decreased platelet count and increased bleeding risk.[1] The pathophysiological mechanism of ITP is complicated, both impaired platelet production and increased platelet destruction contribute to the progression of disease.[1] The pathological antiplatelet antibody produced by B cells is one of the important pathogenic mechanisms. The dysfunction of acquired

immunity in ITP has gained increased attention recently, including excessive survival and the activation of T cells and B cells, impaired regulatory T cells (Tregs) and regulatory B cells (Bregs), and abnormal T cell subsets.[2]

Neutrophils are the most abundant innate immune cells, which have crucial roles in defending against bacterial and fungal infections. Increasing studies suggested that neutrophils are important participants in various autoimmune diseases through the formation of neutrophil extracellular traps (NETs), synthesis of proinflammatory cytokines and direct tissue damage.[3,4] Previous studies have found the neutrophil count, neutrophil activation and NET formation were increased in ITP patients.[5,6] However, the specific pathways of neutrophils participating in the pathogenesis of ITP have yet to be explored.

The B-cell activating factor (BAFF; also known as BLyS, TNFSF13B) is a member of the tumor necrosis factor (TNF) family. It binds with three receptors: BAFF receptor (BAFF-R), transmembrane activator, calcium modulator and cyclophilin ligand interactor (TACI) and B-cell maturation antigen (BCMA).[7] BAFF, mainly secreted by neutrophils and monocytes, is critical for regulating the development, homeostasis and autoreactivity of B cells, as well as the survival and proinflammatory status of T cells.[7,8] There are reports showing that the serum levels of BAFF are increased in active ITP patients.[9–11] However, the source of elevated BAFF in ITP has been rarely studied.

This study aimed to evaluate the expression of BAFF in neutrophils in ITP patients, to study the regulatory factors of neutrophils and the modulation functions of neutrophils on acquired immune cells, thereby providing novel insights into the immune dysregulation as well as therapeutic targets of ITP.

RESULTS

Neutrophils were overactivated and produced elevated levels of BAFF in active ITP patients

A total of 40 healthy controls (median age 55 years, range 21~87 years) and 52 active ITP patients (median age 56 years, range 27~85 years), including 12 ITP patients (median age 52 years, range 21~82 years) requiring treatment and achieving complete remission after treatment were enrolled in this study (Table 1). The neutrophil count was significantly elevated in active ITP patients ($4.66 \pm 2.28 \times 10^9$/L) compared with controls ($3.37 \pm 2.28 \times 10^9$/L, $P < 0.001$) and patients in remission ($3.17 \pm 2.28 \times 10^9$ L^{-1}, $P < 0.001$) (Figure 1a). The mRNA levels of BAFF in neutrophils were significantly increased in active ITP patients (1.44 ± 1.07) compared with those in the controls (0.74 ± 0.51, $P < 0.001$) and patients in remission (0.91 ± 0.48, $P = 0.013$) (Figure 1a). In active ITP patients, there was no differences in neutrophil count (4.59 ± 0.31 *vs* 4.72 ± 0.55 *vs* 4.64 ± 0.52) and mRNA levels of BAFF (0.91 ± 0.22 *vs* 1.34 ± 0.21 *vs* 1.71 ± 0.27) among different platelet count groups (Figure 1b). Immunofluorescence showed the enhanced expression of BAFF and CD11b in neutrophils in ITP patients compared with that in control (Figure 1c). The protein–protein interaction (PPI) network showed the interactions of neutrophil activation and BAFF regulation related proteins (Figure 1d). The mRNA levels of ITGAM (0.94 ± 0.54 *vs* 1.21 ± 0.88, $P = 0.011$), CSF3 (1.62 ± 0.66 *vs* 4.96 ± 1.40, $P = 0.017$), SELPLG (1.01 ± 0.41 *vs* 1.26 ± 0.42, $P = 0.023$) and CCL2 (1.53 ± 0.41 *vs*

Table 1. Characteristics of enrolled individuals

	Control	active ITP	ITP in remission	*P*
Cases	40	52	12	
Age (Years, Median)	55 (21–87)	56 (27–85)	52 (21–82)	NS
Male (%)	50	55.8	41.7	NS
Platelet counts ($\times 10^9$ L^{-1})	242.5 ± 65.6	55.3 ± 36.1	136.5 ± 33.5	< 0.001
Neutrophil count ($\times 10^9$ L^{-1})	3.37 ± 1.00	4.66 ± 2.28	3.17 ± 0.70	0.002
CD62P$^+$ platelets (%)	12.78 ± 13.99	7.39 ± 7.21	20.69 ± 14.18	0.003
PAC-1$^+$ platelets (%)	28.49 ± 27.87	20.12 ± 18.98	48.44 ± 31.62	0.009

NS, no significant; CD62P$^+$ platelets (%) and PAC-1$^+$ platelets (%) were results after TRAP treatment.

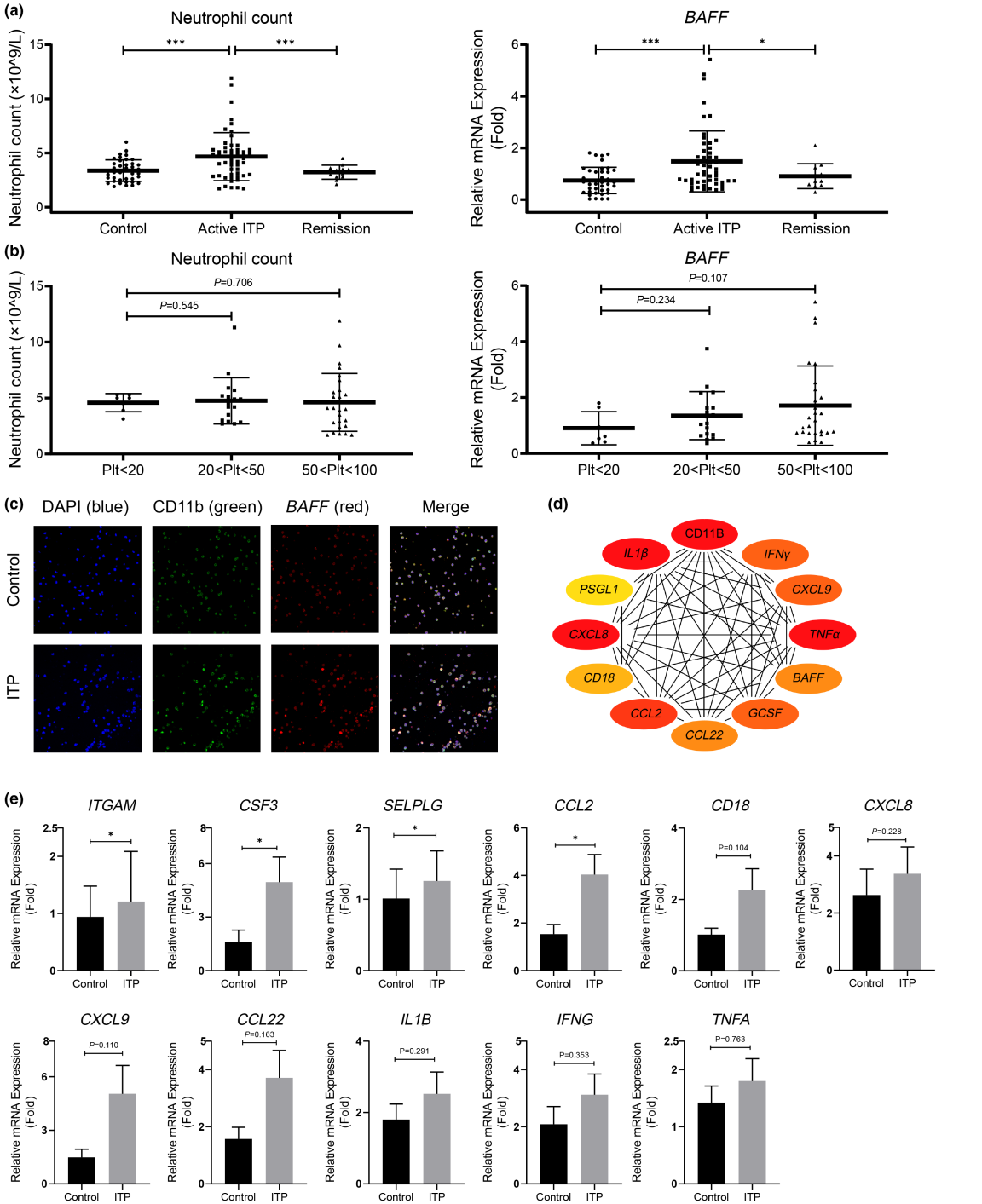

Figure 1. Neutrophil count, activation and BAFF expression were elevated in active ITP patients. **(a, b)** Peripheral neutrophil count and relative mRNA expression of BAFF in neutrophils in healthy controls (n = 40), active ITP patients (n = 52) and ITP patients in remission (n = 12) **(a)** and groups of active ITP patients based on platelet count **(b)**. **(c)** Immunofluorescence staining of neutrophils in healthy controls and active ITP patients (20×). CD11B is used to identify neutrophils. **(d)** Protein-protein interaction (PPI) network of neutrophil activation and BAFF regulation related proteins. The colour intensity of each node is proportional to the interaction degree in the PPI network. **(e)** Relative mRNA expression of *ITGAM*, *CSF3*, *SELPLG*, *CCL2*, *CXCL8*, *CCL22*, *CXCL9*, *CD18*, *IL1B*, *IFNG* and *TNFA* of neutrophils in healthy controls (n = 16) and ITP patients (n = 16). *P < 0.05, ***P < 0.001.

4.04 ± 0.884, $P = 0.046$) in neutrophils were significantly elevated in ITP patients compared with those in controls (Figure 1e).

The activation ability of platelets was attenuated in ITP patient

TRAP activation significantly increased the percentages of $CD62P^+$ platelets (1.31 ± 1.68 *vs* 11.76 ± 12.76, $P < 0.001$) and PAC-1^+ platelets (0.54 ± 1.10 *vs* 16.41 ± 19.11, $P < 0.001$) (Figure 2a). After TRAP activation, the percentages of $CD62P^+$ platelets were significantly decreased in active ITP patients (7.39 ± 7.21) compared with those in controls (12.78 ± 13.99, $P = 0.020$) and patients in remission (20.69 ± 14.18, $P = 0.002$), and PAC-1^+ platelets in active ITP patients (20.12 ± 18.98) were significantly decreased compared with those in patients in remission (48.44 ± 31.62, $P = 0.007$) (Figure 2b). Increase in percentages of $CD62P^+$ platelets upon stimulation of TRAP were significantly attenuated in ITP patients (6.58 ± 7.25) compared with that in the controls (12.58 ± 11.66, $P = 0.007$) and patients in remission (22.58 ± 19.43, $P < 0.001$). The increase in percentages of $CD62P^+$ platelets after TRAP stimulation were more significant in patients in remission (48.29 ± 31.54) than in controls (28.21 ± 24.51, $P = 0.014$) and active ITP patients (19.7 ± 18.95, $P = 0.006$) (Figure 2c).

CD62P induced the generation of BAFF by neutrophils in active ITP patients

Correlation analysis showed the significant correlations between BAFF levels and $CD62P^+$ platelets ($r = 0.385$, $P < 0.001$), PAC-1^+ platelets and platelet count ($r = 0.404$, $P = 0.010$), $CD62P^+$ platelets and PAC-1^+ platelets in active ITP patients ($r = 0.499$, $P = 0.001$) (Table 2). Regression analysis further suggested the linear positive correlations between platelet count and percentages of $CD62P^+$ platelets ($r^2 = 0.11$, $P = 0.016$) (Figure 2d), mRNA levels of BAFF in neutrophils and percentages of $CD62P^+$ platelets ($r^2 = 0.34$, $P = 0.002$) (Figure 2d) in active ITP patients.

To verify the interaction between CD62P and neutrophils, co-culture of neutrophils showed that neutrophils from active ITP patients produced significantly increased BAFF in mRNA levels after co-culturing with platelets (1.00 ± 0.00 *vs* 1.45 ± 0.71, $P = 0.033$) and CD62P (1.00 ± 0.00 *vs* 1.30 ± 0.26, $P = 0.048$), and the effects were inhibited after blocking PSGL1 (1.45 ± 0.71 *vs* 1.06 ± 0.32, $P = 0.037$, 1.30 ± 0.26 *vs* 0.93 ± 0.44, $P = 0.029$) (Figure 2e), while no significant difference was found in neutrophils from normal controls (Supplementary figure 1). ELISA results also showed that neutrophils secreted significantly increased BAFF in the presence of platelets (11.52 ± 4.16 *vs* 25.35 ± 3.77, $P < 0.001$) and CD62P (11.52 ± 4.16 *vs* 20.85 ± 4.21, $P < 0.001$), and the effects were inhibited after blocking PSGL1 (25.35 ± 3.77 *vs* 17.73 ± 6.07, $P < 0.001$, 20.85 ± 4.21 *vs* 11.87 ± 4.73, $P = 0.001$) (Figure 2f).

Neutrophils inhibited the apoptosis of $CD4^+$, $CD8^+$ and $CD19^+$ cells dependent on BAFF levels

Neutrophils from active ITP patients were divided into the high BAFF group or low BAFF group based on the mRNA levels of BAFF (0.74 ± 0.08 *vs* 1.14 ± 0.26, $P < 0.001$) (Figure 3a) and co-cultured with PBMC *in vitro*. The apoptosis of $CD4^+$ T cells, $CD8^+$ T cells and $CD19^+$ B cells was significantly decreased in the high BAFF group compared with that in the low BAFF group (1.37 ± 0.95 *vs* 0.90 ± 0.46, $P = 0.048$, 2.02 ± 1.38 *vs* 1.48 ± 1.03, $P = 0.002$, 9.55 ± 5.77 *vs* 6.43 ± 3.28, $P < 0.001$), and blocking BAFF by belimumab significantly increased the apoptosis in both the low BAFF (1.37 ± 0.95 *vs* 2.19 ± 1.43, $P = 0.032$, 2.02 ± 1.38 *vs* 2.71 ± 1.38, $P = 0.021$, 9.55 ± 5.77 *vs* 114.73 ± 6.88, $P < 0.001$) and high BAFF groups (0.90 ± 0.46 *vs* 1.80 ± 1.04, $P = 0.003$, 1.48 ± 1.03 *vs* 2.36 ± 1.58, $P < 0.001$, 6.43 ± 3.28 *vs* 9.71 ± 5.82, $P = 0.033$) (Figure 3b–d). However, there are no differences in the apoptosis of $CD56^+$ NK cells and $CD14^+$ monocytes (Figure 3e and f).

DISCUSSION

Neutrophils are the first line of defence in adaptive immunity and have gained considerable attention in regulating the development and progression of autoimmune diseases. The present study showed for the first time that neutrophils could affect the acquired immunity by producing excessive BAFF in ITP, thus participating in the pathophysiological of diseases. This immune intolerance could be circumvented by belimumab, a human monoclonal antibody targeting the soluble BAFF. Furthermore, the results showed that the platelet–neutrophil interaction mediated

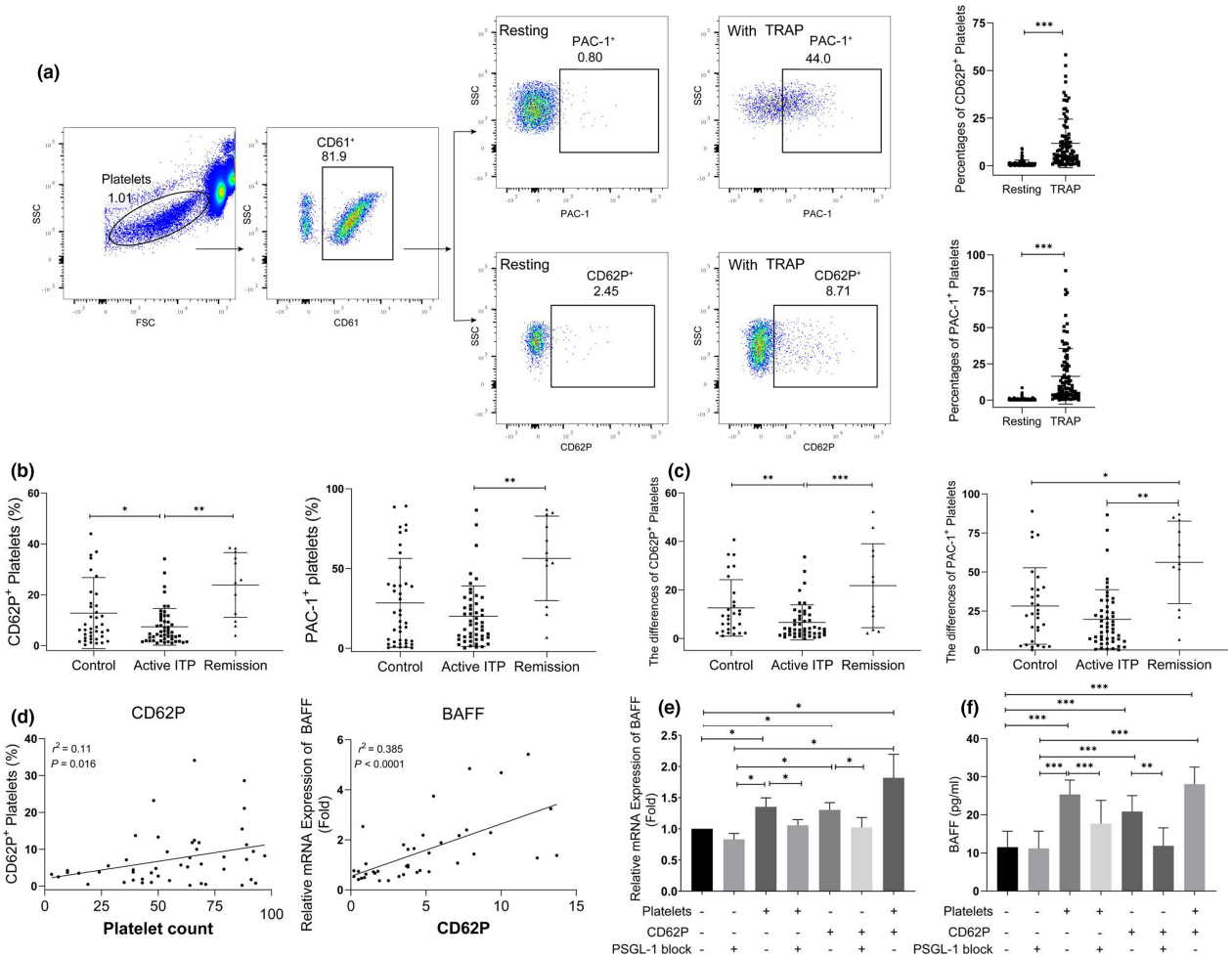

Figure 2. The activation ability of platelets was attenuated in ITP patients and CD62P on platelets induced the generation of BAFF by binding with PSGL1. **(a)** Percentages of CD62P$^+$ platelets and PAC-1$^+$ platelets on resting platelets and TRAP activated platelets. Flow cytometry dot plots show gating for CD62P$^+$ platelets and PAC-1$^+$ platelets. Scatter plots represent the related percentages (n = 95). **(b)** Percentages of CD62P$^+$ platelets and PAC-1$^+$ platelets after TRAP activation in healthy controls (n = 39), active ITP patients (n = 50) and ITP patients in remission (n = 12). **(c)** Differences of CD62P$^+$ platelets and PAC-1$^+$ platelets after TRAP activation in healthy controls (n = 39), active ITP patients (n = 50) and ITP patients in remission (n = 12). **(d)** Linear regression analysis between platelet count and percentages of CD62P$^+$ platelets (left, n = 50), mRNA levels of BAFF in neutrophils and percentages of CD62P$^+$ platelets (right, n = 40) in active ITP patients. **(e, f)** Relative mRNA BAFF levels detected by real-time PCR **(e)** and BAFF concentration detected by ELISA **(f)** in neutrophils after co-culturing with platelets or recombinant human CD62P, with or without PSGL1 blocking antibody (n = 14). *P < 0.05, **P < 0.01, ***P < 0.001.

by CD62P and PSGL1 could stimulate the generation of BAFF in neutrophils.

Neutrophils have been considered to be important regulators of the acquired immune response. The abnormal accumulation and function of neutrophils are associated with chronic inflammation and autoimmune diseases, such as systemic lupus erythematosus, but their role in disease pathogenesis remains to be completely understood.[12,13] Consistent with the previous reports,[5] the current study suggested that the neutrophil count was increased in active ITP patients with an enhanced expression of activation marker, such as CD11B, and C-C chemokines, such as CCL2, and the increase of the neutrophil count was not related to the platelet count. Other studies also reported that neutrophils from active ITP patients expressed higher levels of NETosis.[6] Collectively, these findings suggest that the activation of neutrophils is hyperarousal and present more inflammatory properties in active ITP patients.

Most of the studies regarding the immunoregulation of neutrophils have been

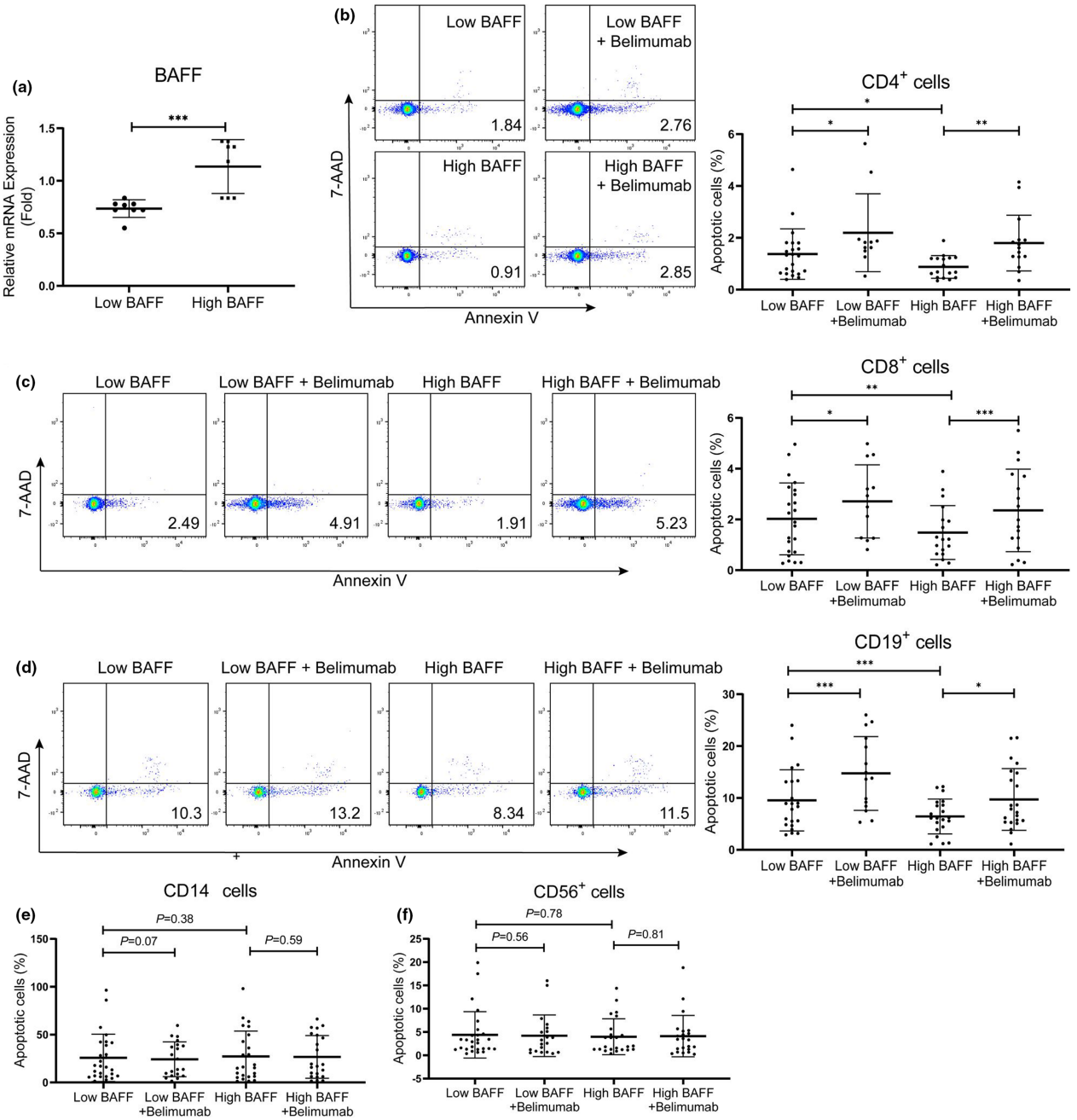

Figure 3. Neutrophils inhibited the apoptosis of $CD4^+$, $CD8^+$ and $CD19^+$ cells dependent on BAFF levels. **(a)** Relative BAFF mRNA expressions of neutrophils in the low BAFF group and high BAFF group (n = 8). **(b–f)** Apoptotic cells were detected in $CD4^+$ **(b)**, $CD8^+$ **(c)**, $CD19^+$ **(d)**, $CD56^+$ **(e)** and $CD14^+$ **(f)** cells. Flow cytometry dot plots show gating for Annexin $V^+7\text{-}AAD^-$ apoptotic cells. (n = 11–27) $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

focused on the NET formation, which is a crucial autoantigen in autoimmune responses in some autoimmune diseases, and its biological properties.[4,14] However, neutrophils are also a major source of cytokines, including BAFF, TNF-related apoptosis-inducing ligand (TRAIL), TNFα and IL-1 receptor antagonist (IL-1RA).[15] Several studies reported that the neutrophils could contribute to excess serum BAFF and promote immune responses in systemic lupus erythematosus, oral lichen planus, and oral cavity cancer.[13,16,17] Our PPI analysis showed that BAFF expression was

Table 2. Correlation analysis of BAFF and platelets

		BAFF	Neutrophil count	Platelet count	PAC-1$^+$	CD62P$^+$
BAFF	*r*		0.078	0.216	0.085	0.354*
	P		0.640	0.180	0.604	0.025
Neutrophil count	*r*	0.078		0.079	−0.295	−0.207
	P	0.640		0.636	0.073	0.213
Platelet count	*r*	0.216	0.079		0.404**	0.150
	P	0.180	0.636		0.010	0.357
PAC-1$^+$ platelets	*r*	0.085	−0.295	0.404**		0.499**
	P	0.604	0.073	0.010		0.001
CD62P$^+$ platelets	*r*	0.354*	−0.207	0.150	0.499**	
	P	0.025	0.213	0.357	0.001	

Spearman analysis, *$P < 0.05$, **$P < 0.01$.

related to the activation of neutrophils. In ITP, the serum BAFF levels in active ITP patients are increased compared with those in the healthy group or remission patients, and restored to normal after treatment such as dexamethasone.[9–11] However, the source and regulation of BAFF have not been fully clarified.

Based on these findings, we found that BAFF produced by neutrophils was increased in active ITP, and its level was restored to normal after effective treatments. These findings indicate that neutrophils are enrolled in the progression of ITP and contribute to excessive levels of BAFF in active ITP. However, there was a not significant elevation in BAFF levels in the higher platelet count group, implying a positive feedback mechanism between BAFF and platelet count, or there are sources other than neutrophils for the excessive BAFF production in patients with severe thrombocytopenia. Studies have found a higher BAFF mRNA expression in mononuclear cells in ITP.[11,18] Taken together, it is suggested that several cell types may correlate with elevated BAFF in ITP patients, which warrants further exploration.

The regulation of neutrophil-derived cytokines is controlled by complicated mechanisms. Platelet–neutrophil interactions mediate the inflammatory process and intracellular signalling *via* downstream signals such as tyrosine kinases and consecutive integrin activation.[19,20] Zhou *et al*.[11] reported that there was a weak correlation between the platelet count and serum BAFF in ITP. These findings strongly suggest the certain relations between BAFF expression and platelet–neutrophil interactions. The results of regression analysis and co-culture experiment of the study indicated that there is a higher BAFF expression by neutrophils when they are co-cultured with platelets. There are several classes of receptors expressed on the surface of neutrophils, including G-protein-coupled seven-transmembrane receptors, Fc-receptors and adhesion molecules like selectins/selectin ligands and integrins.[21] The combination of CD62P (also known as P-selectin) and PSGL1 constitutes one of the most critical physical interactions between platelets and neutrophils.[21] In our previous studies, the expression of CD62p in platelets in the resting state was higher than that in normal people.[22] In this study, after TRAP stimulation, the expression of CD62p in platelets in ITP patients was lower than that in normal people, which might be because the platelet function was impaired in ITP disease states. PPI analysis showed that the expression of BAFF has connection with PSGL-1. In the present study, PSGL1, a cell adhesion molecule binding with P-, E- and L-selectins, was found increased on neutrophils in ITP patients. Supplementation of CD62P independently could enhance BAFF expression and secretion in neutrophils, while blocking PSGL1 could reduce this effect. These suggest that the CD62P-PSGL1 combination plays a significant role in the BAFF secretion in neutrophils. Studies found CD62P, through binding to PSGL1, could promote activation, NET formation, rolling, and recruitment to inflammatory sites of neutrophils.[23–25] The initial binding of CD62P and *PSGL1* could be stabilised by several other receptors, and then provokes a series of cascades to activate and recruit more platelets and neutrophils, including the releasing of granules, producing various cytokines and molecules, such as thromboxane A2 (TXA2), CXCL4, von Willebrand factor (vWF) and high-mobility group box 1 protein (HMGB1).[26,27] These processes suggest that the combination of PSGL-1 and CD62P could be strengthened once it

occurs, which may enable the platelets with relatively low CD62P expression to activate more neutrophils and response to stimulation rapidly. Since NETs can induce the secretion of BAFF,[14] this may be a potential pathway for the downstream signalling of CD62P stimulated BAFF secretion.

In ITP patients, abnormally elevated BAFF level was found to aggravate disease by promoting the proliferation of $CD19^+$ B cells and $CD8^+$ T cells,[28,29] increasing the apoptosis of platelets, stimulating the production of antibodies by B Cells,[30] and interacting with the monocyte-derived dendritic cells.[30] Thus, the pivotal role of BAFF in immune regulation highlights the feasibility of BAFF as a therapeutic target for ITP. The co-culture experiments of the study also showed that neutrophils affect apoptosis of T cells and B cells in a BAFF-dependent manner. The inhibition of BAFF by belimumab effectively interrupted the effects of neutrophils, suggesting that blockade of BAFF might be a promising therapeutic approach for the management of ITP. A prospective phase IIb trial in ITP reported notable efficacy and safety of a combined rituximab and belimumab regimen in persistent or chronic adults ITP patients, for that 80% patients achieved an overall response without serious complications after 5 weeks of treatment.[31] Moreover, BAFF blockade therapy such as belimumab has shown prominent therapeutic effect in systemic lupus erythematosus and other autoimmune diseases such as rheumatic arthritis and sjögren's syndrome.[32–35] Therefore, multicentre and large-scale trails are anticipated to investigate the therapeutic application of belimumab in ITP.

In conclusion, the current study highlights the participation of neutrophils in the pathophysiological process in ITP, and its regulatory role in the adaptive immune response by secreting BAFF. In addition, the study also reveals that the platelet–neutrophil interaction mediated by CD62P and PSGL1 could modulate BAFF production of neutrophils. Moreover, this study provides evidence for the application of belimumab as a potential treatment for the therapy of ITP, which is worth further investigation.

METHODS

Patients and healthy controls

From October 2020 to August 2021, a total of 52 ITP patients, defined as active ITP patients,[28] with a median age of 56 ranging from 27 to 85 years old were enrolled according to the ITP diagnosis criteria proposed by an international working group.[36] Patients were excluded if (1) receiving platelet transfusion within seven days or ITP-related medication within one month; (2) combining with active or chronic infection, trauma, diabetes, hypertension, cardiovascular diseases, thrombosis, malignant tumor and receiving any antiplatelet therapies. Among 52 active ITP patients, there were 12 ITP patients with platelet counts less than 30×10^9/L or identified comorbidities for bleeding requiring treatment and achieving complete remission (platelet counts more than 100×10^9/L) after treatment. Forty healthy volunteers were recruited as normal controls. The study was approved by the institutional review board of Zhongshan Hospital and Jinshan Hospital, Fudan University. Written informed consent was obtained from each patient prior to the enrollment.

Isolation of platelets, neutrophils and peripheral blood mononuclear cells (PBMC)

Fresh peripheral venous blood was collected and centrifuged (800 rpm at room temperature for 10 min) within one hour. The upper layer of platelet-rich plasma was then centrifuged (3000 rpm at room temperature for 10 min) to obtain platelets and the lower layer of blood cells was resuspended in phosphate buffered saline without Ca^{2+} and Mg^{2+} (DPBS). Then, 3 mL of LymphoPrep (Alere Technologies AS, Oslo, Norway) was carefully layered on top of 3 mL PolymorphPrep (Alere Technologies AS, Oslo, Norway) in a 15-mL centrifuge tube, and the diluted blood cell suspension was overlayed on top of this discontinuous density gradient for gradient centrifugation (2000 rpm at room temperature for 20 min without brake). After centrifugation, there were five layers from top to bottom in the tube: DPBS, PBMC, Ficoll–Hypaque, neutrophils, PolymorphPrep and red blood cells. Neutrophils and PBMC were collected and washed twice for further experiments. The purity of neutrophils was more than 90% detected by flow cytometry.

Cell culture

For the co-culture of neutrophils and platelets, neutrophils isolated from ITP patients were cultured with or without platelets from ITP patients for 12 h, and the ratio of platelets to neutrophils was 10:1. Recombinant human CD62P protein (1 $\mu g\ mL^{-1}$, SinoBiological, Beijing, China) and anti-human PSGL1 antibody (1 $\mu g\ mL^{-1}$, Biolegend, San Diego, USA) were selectively added based on experimental groups. Neutrophils were isolated after coculture for real-time PCR and culture supernatants were collected for the ELISA test. For the co-culture of neutrophils and PBMC, half of the neutrophils from different ITP patients were cultured with PBMC from the same ITP patients at 1:1 ratio for 24 h. Another half of neutrophils were used for detection of BAFF mRNA levels to divide neutrophil–PBMC coculture systems into the high BAFF group and low BAFF group. All cells were incubated in an IMDM medium (Gibco, New York, USA) with 1% penicillin/streptomycin (Gibco) and 20% heat-inactivated fetal bovine serum (Gibco).

Real-Time PCR

RNA was extracted from neutrophils using TRIZOL reagent (Takara, Kyoto, Japan) according to the manufacturer's instructions and reverse-transcripted into cDNA using a cDNA synthesis kit (Yeason, Shanghai, China). The mRNA expressions of *BAFF*, *CD11B*, *GCSF*, *PSGL1*, *CCL22*, *IFNG*, *CCL2*, *CXCL8*, *CXCL9*, *IL1B* and *TNFA* were quantified using the SYBR Premix Ex Taq (Vazyme, Nanjing, China), with actin expression as a control. Primer sequences are shown in Supplementary table 1. Samples were analysed in triplicate, and $2^{-\Delta\Delta Ct}$ was used to calculate the fold change of mRNA expression.

Flow cytometry

For the evaluation of PAC-1 and CD62P on platelets, anticoagulated whole blood was immediately stained (referred as resting platelets) or stimulated by thrombin receptor activating peptide (TRAP, Merck KGaA, Darmstadt, Germany) for 20 min (referred as platelets with TRAP) and then stained with the following anti-human antibodies (Biolegend, San Diego, USA): CD61-Percp/Cy5.5, CD62P-PE and PAC-1-FITC. This procedure was gently operated at room temperature and completed within 1 h after blood withdrawal.

For the apoptosis of PBMC, cells were collected and stained with the following anti-human antibodies or fluorescent dye (Biolegend, San Diego, USA) according to the manufacturer's instructions: Annexin V-FITC, 7-AAD, CD19-PE, CD4-PE/CY7, CD8-APC, CD14- APC/Cyanine7 and CD56-Brilliant Violet 510. The acquisition was performed on a FACS AriIII flow cytometer (BD Biosciences, New York, USA) and then analysed using Flowjo software version 10.0.1. Annexin V^+ 7-AAD^- cells were considered as apoptotic cells.

Immunofluorescence

Neutrophils from peripheral blood of ITP patients were fixed with 4% paraformaldehyde (Yeason, Shanghai, China) and were dehydrated, agarose-embedded and sliced up. Cells-containing slides were stained with DAPI (Abcam, Cambridge, UK) for nucleus, anti-human CD11B (Abcam) for neutrophils and anti-human BAFF antibody (Abcam), and they were subsequently treated with fluorescence secondary antibodies (Abcam). Fluorescence images were taken with an LSM confocal microscope (FV3000; Olympus, Tokyo, Japan).

Statistical analysis

The proteins considered to be associated with neutrophil activation and BAFF regulation were generated to a protein–protein interaction (PPI) network through the STRING database (https://string-db.org/) with a clustering coefficient > 0.35, and the PPI network was visualised in Cytoscape (Version 3.7.1) software.

All analyses were performed using SPSS software (version 25.0; SPSS Inc., USA). Data are expressed as the mean $\pm$ SD. Normality was assessed by the Shapiro–Wilk test. Pairwise comparison was analysed by the Student's *t*-test for normally distributed data and the Mann–Whitney *U*-test for non-normal distributed data. Multi-group comparison was determined by the one-way ANOVA or Welch ANOVA as appropriate. Correlation analysis was performed by Spearman correlation analysis. Two-sided *P*-values < 0.05 were considered statistically significant.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Pengcheng Xu: Data curation; investigation; methodology; resources; writing – original draft; writing – review and editing. **Xia Shao:** Data curation; formal analysis; methodology; writing – original draft. **Yang Ou:** Data curation; formal analysis; methodology; resources; software. **Yanxia Zhan:** Data curation; formal analysis; methodology; resources. **Lili Ji:** Data curation; formal analysis; investigation; methodology. **Xibing Zhuang:** Methodology; resources; software; validation; visualization. **Ying Li:** Data curation; methodology. **Yanna Ma:** Data curation; methodology; software; validation; visualization. **Duojiao Wu:** Methodology; supervision; visualization. **Tiankui Qiao:** Resources; software; supervision. **Xiangdong Wang:** Investigation; project administration; supervision. **Hao Chen:** Conceptualization; funding acquisition; methodology; project administration; resources; supervision; writing – review and editing. **Yunfeng Cheng:** Conceptualization; funding acquisition; investigation; project administration; writing – review and editing.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was in accordance with the ethical standards formulated in the Helsinki Declaration and was approved by the respective local Medical Ethics Committees of Zhongshan and Jinshan Hospital of Fudan University. Written informed consent was obtained from each patient before being included in the study.

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Supporting Information

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Graphical Abstract

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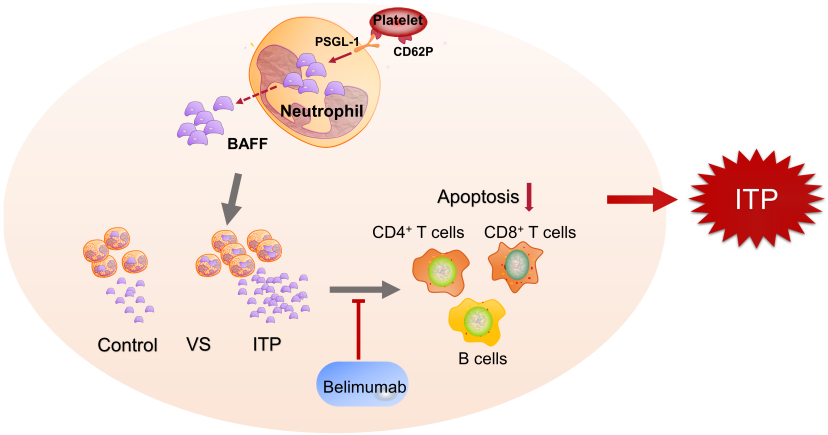

Immune thrombocytopenia (ITP) is an autoimmune disease caused by multifaceted immune dysregulation. In this study, we found that neutrophils produce excessive B-cell activating factor (BAFF) in ITP under the stimulation of CD62P on platelets, which could influence the apoptosis of cells for acquired immunity. Furthermore, we found that targeting BAFF by belimumab may be a potential therapy for ITP.