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BAFF and BAFF-R-Fc in ITP

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Abstract

Elevated level of B-cell activating factor (BAFF) has been implicated in the pathogenesis of some autoimmune diseases. Blockade of receptor and ligand binding by decoy receptor has demonstrated clinical benefit in both oncological and immunological diseases. In this report, we have detected plasma BAFF and BAFF mRNA expression in immune thrombocytopenia (ITP) patients by ELISA and real time quantitative PCR (RT-PCR). The effects of recombinant human BAFF (rhBAFF) and BAFF-R-Fc fusion protein (BR3-Fc) on B cells, T cells, platelets, secretion of IFN-gamma (IFN-γ) and IL-4 were measured by flow cytometry and ELISA. Patients with active disease had higher levels of plasma BAFF and BAFF mRNA than patients in remission and controls. In *in vitro* assays, rhBAFF promoted the survival of $CD19^+$ and $CD8^+$ cells, increased the apoptosis of platelets and the secretion of IFN-γ. BR3-Fc successfully corrected the above effects of rhBAFF. These findings suggest that BAFF may play a pathogenic role in ITP by promoting the survival of $CD19^+$ and $CD8^+$ cells, increasing the apoptosis of platelets and the secretion of IFN-γ. Blockade of BAFF by BR3-Fc might be a promising therapeutic approach for ITP.

Introduction

Immune thrombocytopenia (ITP) is an autoimmune disorder in which the patients' immune system is activated by platelet autoantigens resulting in immune-mediated platelet destruction and/or suppression of platelet production.[1] The autoantibodies produced by autoreactive B cells against self-antigens, specifically IgG antibodies against GPIIb/IIIa and /or GPIb/IX, are considered to play a crucial. role.[2] In addition, several abnormalities involving the cellular mechanisms of immune modulation, such as the Th1 bias,[3, 4] the decreased number or defective suppressive function of regulatory T cells[5, 6, 7] and the platelet destruction by cytotoxic T cells (CTL),[8-10] have been described. The cause for these abnormalities remains unknown. Moreover, the treatment regimens for ITP including glucorticosteroids (GCs), intravenous immunoglobulin G, anti-D and splenectomy are not always effective, and only one-third of adult patients achieve long-term remission.

B-cell activating factor (BAFF) (also known as BlyS, TALL-1, THANK, zTNF4 and TNFSF13B) belonging to the family of tumor necrosis factor (TNF) ligands is critical for the maintain of normal B-cell development, homeostasis and autoreactivity [11, 12] and T cell costimulation.[13-15] In addition, it also augments certain Th1-associated inflammatory responses.[16] BAFF binds to three receptors: B-cell maturation antigen (BCMA, TNFRSF17), transmembrane activator and calcium-modulating cyclophilin ligand (CAML) interactor (TACI, TNFRSF13B) and BAFF receptor (BR3/BAFF-R, TNFRSF13C) .[17, 18] BR3, identified as the crucial receptor for B-cell survival, is expressed on a wide range of B-cell subsets, including immature, transitional, mature, memory and germinal center B cells, as well as on plasma cells.[19] Furthermore, BAFF binding to BR3 on T cells has been shown to costimulate T-cell proliferation both *in vitro* and *in vivo*.[15]

Several lines of evidence suggested that BAFF may play an important role in autoimmunity. Autoantigen-binding B cells may have an increased dependence on the BAFF survival signal. [20] In addition, elevated BAFF plasma level was observed in many patients with autoimmune diseases such as rheumatoid arthritis (RA),[21] systemic lupus erythematosus (SLE),[22] Sjögren's syndrome (SS),[23] and multiple sclerosis (MS).[24] Inhibition of BAFF signaling is a potentially therapeutic option for treatment of B cell-mediated autoimmune conditions. Data from animal tests and clinical trials had proved that blockade of BAFF by blocking reagents (TACI-Ig, BAFF-R-Ig, BR3-Fc) was effective therapeutic approach for some autoimmune diseases.[25-27]

In our study, we focused on the effects of BAFF and BR3-Fc in ITP, and found rhBAFF could promote the survival of $CD19^+$ and $CD8^+$ cells, increase the apoptosis of platelets and the secretion of IFN-γ while BR3-Fc successfully corrected the above effects of rhBAFF, suggesting a possible role of BAFF in the pathogenesis of ITP.

Materials and Methods

Patients

Forty-five patients diagnosed with ITP were selected for detection of plasma BAFF and BAFF mRNA. Of these patients, twenty-five patients (15 females and 10 males, median 41 years; platelets range $1\text{-}36\times10^9$/L, median 15×10^9/L) were active ITP patients with platelets $< 50\times10^9$/L who had not been treated with GCs for at least one month prior to sampling, whereas 20 patients were in remission with normal platelets counts (12 females and 8 males, median 37 years; platelets range $99\text{-}299\times10^9$/L, median 191×10^9/L) (Supplementary table1). Twenty-four healthy controls matched for sex and age with the study population were voluntary blood donors. Peripheral blood mononuclear cells (PBMCs) and platelets from additional eighteen ITP patients with active disease (10 females and 8 males, median 40 years; platelets range $9\text{-}49\times10^9$/L, median 25×10^9/L) (Supplementary table2) were selected for detection of apoptosis on $CD19^+$, $CD4^+$, $CD8^+$ cells and secretion of cytokines, patient 1 to patient 12 in Supplementary table 2 were also used for detection of apoptosis on platelets. Fifteen healthy controls (9 females and 6 males, median 41 years; platelets range $157\text{-}297\times10^9$/L, median 218×10^9/L) were used for cell culture. Enrollment took place between January 2008 and August 2009 at the Department of Hematology, Qilu Hospital, Shandong University. All of the cases met the diagnosis criteria of ITP as previously described.[28] Informed consent was obtained from each patient and healthy control in accordance with the Declaration of Helsinki. Ethical approval for the study was obtained from the Medical Ethical Committee of Qilu Hospital, Shandong University.

Preparation of PBMCs and platelets

PBMCs were isolated from heparinized blood using 1.077g/ml Ficoll-Hypaque (Invitrogen, Carlsbad, CA) gradient centrifugation (2000 rpm for 20 min, 20°C). The isolated PBMCs were washed twice with 0.9% NaCl then resuspended and adjusted to 1×10^6 PBMCs /ml for cell culture and 1×10^6 additional PBMCs were stored at -80°C for RT-PCR.

Autologous platelets were separated from heparinized blood by centrifugation at 200g for 15 min at 20°C. The platelet-rich plasma (PRP) was then centrifuged at 800g for 10 min and the platelet pellet was washed once in 0.9% NaCl and resuspended, adjusted to 1×10^7platelets /ml for cell culture.

BAFF, IFN-γ and IL-4 determination by ELISA

The level of plasma BAFF (R&D Systems, Minneapolis, MN, USA) was assayed by ELISA following the manufacturer's recommendations.

The levels of IFN-γ and IL-4 in supernatant of culture were assayed by ELISA (Bender MedSystems, Burlingame, CA, USA). Briefly, 1×10^6 PBMCs /well with 1×10^7 autologous platelets/well were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 10μg/ml phytohemagglutinin (PHA) (Sigma, St. Louis, MO, USA) in 24-well plate (1ml final vol) with rhBAFF (R&D Systems, Minneapolis, MN, USA), combination of rhBAFF and BR3-Fc (Genetech, South San Francisco, CA , USA) at 37℃ with 5% CO_2. Cells were harvested after 72 hours and stored at -80°C for use. The protein levels of IFN-γ and IL-4 in supernatant were determined by ELISA.

Determination of the expression of BAFF mRNA

For reverse transcription, the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA. RNA was converted into cDNA using the PrimeScript™

RT Reagent Kit (Perfect Real Time) (Takara, Kyoto, Japan) according to the manufacturer's instructions. Multiplex RT-PCR was performed for BAFF and the endogenous control (β-actin) on an ABI PRISM_7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) by using SYBR Green (Toyobo, Osaka, Japan) as a double-strand DNA-specific binding dye. The primers for all mRNA assays were intron-spanning. The PCR reactions were cycled 40 times after initial denaturation (95°C, 5 min) with the following parameters: denaturation at 95°C for 15s; annealing at 60°C for 15 s; extension at 72°C for 35s, with temperature transition rates of 20°C /s. The primers for BAFF and β-actin are as follows: BAFF-Forward: AAGACCTACGCCATGGGACATC,BAFF-Reverse:TCTTGGTATTGCAAGTTGG AGTTCA; β-actin–Forward: TTGCCGACAGGATGCAGAA; β-actin–Reverse: GCCGATCCACACGGAGTACT.

We used the comparative Ct method (Using arithmetic formulae) for relative quantification of mRNA according to relative expression software tool (REST©).[29] The amplification efficiency between the target (BAFF) and the reference control (β-actin) was compared in order to use the delta delta Ct (ΔΔCt) calculation.

The effects of rhBAFF and/or BR3-Fc on apoptosis of $CD19^+$ cells, $CD4^+$ cells, $CD8^+$ cells and autologous platelets by flow cytometry

1×10^6 PBMCs/well with 1×10^7 autologous platelets/well were cultured in RPMI 1640 medium supplemented with 10% FCS in 24-well plate (1ml final vol) with rhBAFF (20ng/ml), combination of rhBAFF (20ng/ml) and BR3-Fc (100μg/ml) at 37℃ with 5% CO_2. PBMCs were harvested after 48 hours and incubated with 20 μl PE-Cy5-conjugated CD19 , PE-conjugated CD8 or PE-Cy5-conjugated CD4 (BD Biosciences, San Jose, CA, USA) for 30 minutes. Cells were washed and incubated with 5 μl FITC-conjugated annexin V (Invitrogen) for 15 minutes and were analyzed within 1 hour by FACS.

In order to investigate the effects of rhBAFF and/or BR3-Fc on the apoptosis of autologous platelets, 1×10^6 PBMCs/well alone were cultured with rhBAFF, combination of rhBAFF and BR3-Fc in 24-well plate for 48 hours, and then were added 1×10^7 autologous platelets/well. After 4 hours, platelets were harvested and incubated with 20 μl PE-Cy5-conjugated CD41 (BD Biosciences) for 30 minutes, washed, incubated with 5 μl FITC-conjugated annexin V and were analyzed within 1 hour by FACS. The assay was to measure annexin V binding to detect membrane phosphatidylserine (PS) exposure. While normal platelet activation also increases annexin V binding, in order to accurately detect platelet apoptosis, we also measured apoptosis of platelets in additional nine ITP patients by mitochondrial membrane potential assay kit with JC-1 which is a marker of mitochondrial activity (Beyotime, Nantong, Jiansu, China). In normal undamaged nucleate cells, mitochondria has a high mitochondrial transmembrane potential ($\Delta\Psi_m$). Breakdown of $\Delta\Psi_m$ is characteristic of early apoptosis. As in other cells, $\Delta\Psi_m$ in platelets can be measured by cell-penetrating lipophilic cationic fluorochrome JC-1. Cells containing forming J-aggregates have high ΔΨm, and show red fluorescence (FL2). Cells with low ΔΨm are those in which JC-1 maintains (or re-acquire) monomeric form, and showing green fluorescence (FL1). Depolarization of $\Delta\Psi_m$ was measured by JC-1 which accumulates in mitochondrial matrix, driven by $\Delta\Psi_m$, and expressed as an increase of green to red (G/R) fluorescent ratio reflecting the transformation of JC-1 aggregates into monomers when mitochondrial membrane becomes depolarized.[30]

Anti-platelet autoantibody determination

All plasma samples and cell culture supernatant were stored at -20°C prior to use. The specific anti-platelet GPIIb/IIIa and/or GPIb/IX autoantibodies were analyzed by modified monoclonal antibody specific immobilization of platelet antigens (MAIPA) which was carried out as previously described in detail by Hou et al.[31]

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical significance was determined by ANOVA. All tests were performed by SPSS 13.0 system. P value less than 0.05 was considered statistically significant.

Results

Elevated levels of plasma BAFF and BAFF mRNA in active ITP patients

Figure 1A shows the plasma BAFF levels of different groups. The level of plasma BAFF in ITP patients with active disease was significantly higher (mean ± SD, 593.1±219.0 pg/ml) than that in patients in remission (432.5±121.4pg/ml, P<0.05) and controls (454.4±132.5pg/ml, P<0.05). No significant difference between patients in remission and healthy controls was found (P >0.05).

Using the REST software, the data were presented as the fold change in gene expression normalized to an endogenous reference gene and relative to healthy controls. The relative amount of BAFF mRNA in patients with active disease was increased 3.1 and 2.5-fold compared to patients in remission (P<0.01) and healthy controls (P<0.01), respectively. Of all the subjects, there was no significant difference between patients in remission and healthy controls (P >0.05) (Figure 1B).

Effects of rhBAFF and/or BR3-Fc on apoptosis of peripheral $CD19^+$, $CD4^+$ and $CD8^+$ cells

We enrolled 18 active ITP patients and 15 healthy controls for cell culture. RhBAFF significantly decreased the annexin V% of $CD19^+$ cells in ITP patients but not in controls. BR3-Fc corrected the effect of rhBAFF on apoptosis of $CD19^+$ cells (Figure 2A).

Compared with healthy controls, the annexin V% of $CD8^+$ cells was significantly decreased in ITP patients (ITP: 6.5±3.2%, controls: 10.5±2.7%, P<0.05). RhBAFF significantly decreased the annexin V% of $CD8^+$ cells in both ITP patients and controls. BR3-Fc corrected the effect of rhBAFF on apoptosis of $CD8^+$ cells only in ITP patients. The annexin V% on $CD8^+$ cells in ITP patients in groupⅠ(rhBAFF 0ng/ml), groupⅡ(rhBAFF 20ng/ml) and groupⅢ(rhBAFF+BR3-Fc) were 6.5±3.2%, 4.4±2.2% and 6.3±2.9%, respectively, The annexin V% on $CD8^+$ cells in controls in groupⅠ, groupⅡ and groupⅢ were 10.5±2.7%, 8.3±3.2% and 8.9±4.0%, respectively (Figure 2B).

There was no significant effect of rhBAFF on annexin V% of $CD4^+$ cells in both ITP patients and controls (P>0.05). The annexin V% of $CD4^+$ cells in ITP patients in groupⅠ, groupⅡ and group Ⅲ were 8.6±4.5%, 5.3±1.8% and 8.2±3.8%, respectively.

Effects of rhBAFF and/or BR3-Fc on apoptosis of autologous platelets

Since rhBAFF significantly promoted the survival of $CD8^+$ T cells which could destruct platelets by cytotoxic T-lymphocyte-mediated platelet lysis, we investigated the effects of rhBAFF and/or BR3-Fc on the apoptosis of platelets. RhBAFF significantly increased apoptosis of platelets in ITP patients but not in controls. BR3-Fc corrected the effect of rhBAFF on apoptosis of platelets (Figure 2C). In order to further confirm the results, we also measured the apoptosis of platelets by mitochondrial membrane potential assay kit with JC-1 which was a more precise method for detection of platelet apoptosis. Similar results were found. Figure 3 represents the apoptosis of platelets in different groups in a typical ITP patient measured by JC-1.

Effects of rhBAFF and/or BR3-Fc on secretion of cytokines by PBMCs

The levels of IFN-γ and IL-4 in supernatant were measured by ELISA. RhBAFF promoted the secretion of IFN-γ in the presence of PHA (10μg/ml) (P<0.05) in ITP patients but not controls, and combination of BR3-Fc and rhBAFF reduced the level of IFN-γ compared with group rhBAFF (20ng/ml) (P<0.05). The mean ± SD of groupⅠwas 74.0±12.5 pg/ml, and it increased to 95.1±25.7pg/ml in groupⅡ, and reduced to 82.4±17.4pg/ml in groupⅢ, similar to that in groupⅠ(Figure 2D). There was no detectable level of IFN-γ when incubating cells without PHA. The level of IL-4 was below the detectable limit of the assay used.

Discussion

In this study, we have demonstrated that the levels of plasma BAFF and BAFF mRNA were elevated in active ITP patients, while in patients in remission, normal levels of plasma BAFF and BAFF mRNA expression were observed. These results indicated BAFF was correlated to disease activity in ITP patients. Elevated plasma BAFF level has been detected in other autoimmune diseases, such as SLE and RA which are primarily mediated by autoreactive B-cell and T-cell clones.[21, 22] ITP is an acquired autoimmune disease which is mediated by autoreactive B-cell and T-cell clones. The immune response in the pathogenesis of the disorder involves a complex interaction between antigen presenting cells (APCs), T cells and B cells. The exact mechanism underlying the relationship between excess BAFF and immune dysfunction is generally not known in ITP.

BAFF is a crucial homeostatic cytokine for B cells that is upregulated during inflammation and links adaptive with innate immunity. BAFF has been shown to enhance the expression of CD19 and mediate the maturation of autoreactive B cells.[32, 33] Pers JO et al [34] had reported that high levels of BAFF were associated with the presence of autoantibodies (anti-double-stranded DNA antibodies in SLE, anti-SSA antibodies in pSS, and rheumatoid factors in RA). However, some studies found that BAFF levels did not correlate with autoantibody titers in SLE [35], BAFF stimulation of B-cells may contribute to SLE by other mechanisms than autoantibody production. In our study, no association was found between the levels of BAFF and anti-platelet autoantibodies, and addition of rhBAFF didn't promote the production of autoantibodies in vitro. These findings suggested that excessive BAFF may not directly promote the production of anti-platelet autoantibodies but may play a role by other mechanisms than autoantibody production in ITP patients.

To further illuminate the mechanisms between excess BAFF and immune dysfunction in ITP, we detected the effects of rhBAFF on the apoptosis of $CD19^+$, $CD4^+$ and $CD8^+$ cells, and found rhBAFF could not only promote the survival of $CD19^+$ cells but also promote the survival of $CD8^+$ cells in ITP patients. Among 18 ITP patients, 14 of them had more decreased annexin V% of $CD19^+$ and $CD8^+$ cells in group rhBAFF20ng/ml than in group rhBAFF0ng/ml. BR3-Fc corrected the effects of rhBAFF on annexin V% of $CD19^+$ and $CD8^+$ cells in these 14 patients. It's of interest to note that the annexin V% of $CD8^+$ cells in ITP patients was significantly decreased compared with healthy controls, more importantly, rhBAFF remarkably promoted the survival of $CD8^+$ cells in ITP patients. Further study showed that the apoptosis of platelets increased in ITP patients when autologous platelets were incubated with PBMCs from the same patient after rhBAFF was added, while the apoptosis of platelets did not increase when without PBMCs, indicating BAFF may contribute to thrombocytopenia partially by cell-dependent platelets destruction in ITP. Among the 18 ITP patients of our experiment, we investigated the effect of rhBAFF on apoptosis of platelets in 12 patients. Nine of them had more increased apoptosis of platelets in group rhBAFF 20ng/ml than in group rhBAFF0ng/ml. In these 9 patients, 6 patients (67%) were negative for anti-platelet GPIIb/IIIa and GPIb /□ autoantibodies. The results confirmed our former finding that increasing cytotoxic T-lymphocyte-mediated platelet lysis was the predominant cause of thrombocytopenia in ITP patients without platelet autoantibodies.[9, 10] In addition, we also found rhBAFF could increase the production of IFN-γ but had no obvious effect on the secretion of IL-4 in vitro experiment, which was consistent to early studies that there was a typical Th1-mediated responses and reduced Th2-mediated responses in BAFF Transgenic mice.[16]

ITP is a heterogeneous disease, besides humoral immune abnormalities，a number of T-cell abnormalities have been demonstrated in patients with ITP, including Th1 bias, the inhibition of autologous megakaryocyte apoptosis by CD8+ T cells and cell-mediated cytotoxic lysis of platelets by CTLs.[3, 4, 8-10, 35] Our results indicated that elevated BAFF not only was involved in humoral immune abnormalities by promoting the survival of B cells, but may also be involved in the cellular immunity abnormalities by promoting the survival of T cells.

BR3-Fc, a fully human fusion protein of the extracellular domain of human BAFF-R with the Fc of human IgG1, is a selective BAFF blockade which could block the interaction of BAFF with all its three receptors. In this study, we demonstrated that BR3-Fc could significantly promote the apoptosis of $CD19^+$ cells in ITP and block the BAFF-mediated survival of B cells. In addition to promote the apoptosis of $CD8^+$ cells, BR3-Fc also inhibited the secretion of IFN-γ and the apoptosis of platelets. BAFF blockade could result in B-cell reduction in animal models.[36, 37] Recent clinical trials with BAFF blockade have shown clinical benefit in SLE and RA.[38, 39] Treatment of lupus-prone NZB/WF1 mice with both BR3-Fc and TACI-Ig significantly decreased the proteinuria and glomerular damage in these mice.[40] These findings in conjunction with the evidence of in vitro test and joint BAFF elevations in patients with autoimmune disease offer further support to the contention that blockade of BAFF signaling may be of therapeutic benefit in a variety of autoimmune diseases， such as SLE and RA.

In summary, BAFF is elevated in ITP patients with active disease, and excessive BAFF may rescue autoreactive B and T cells from apoptosis. Increased survival of $CD8^+$ T cells may promote the apoptosis of platelets through CTL-mediated platelet lysis. BR3-Fc, a selective BAFF blockade, could successfully correct the effects of rhBAFF by promoting the apoptosis of $CD19^+$ and $CD8^+$ cells and inhibiting secretion of IFN-γ. Blockade of BAFF by BR3-Fc is promising therapeutic approach for ITP especially those with active disease.

Authorship

Contribution: Z.X. performed research, analyzed data and wrote the manuscript, S.Y. contributed vital new reagents, designed and performed research, and wrote the manuscript, P.J. performed research and wrote the manuscript, S.N. performed research, G.C. analyzed data, Q.P. analyzed data, J.X. analyzed data, H.M. designed the research and wrote the manuscript.

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Figure legends

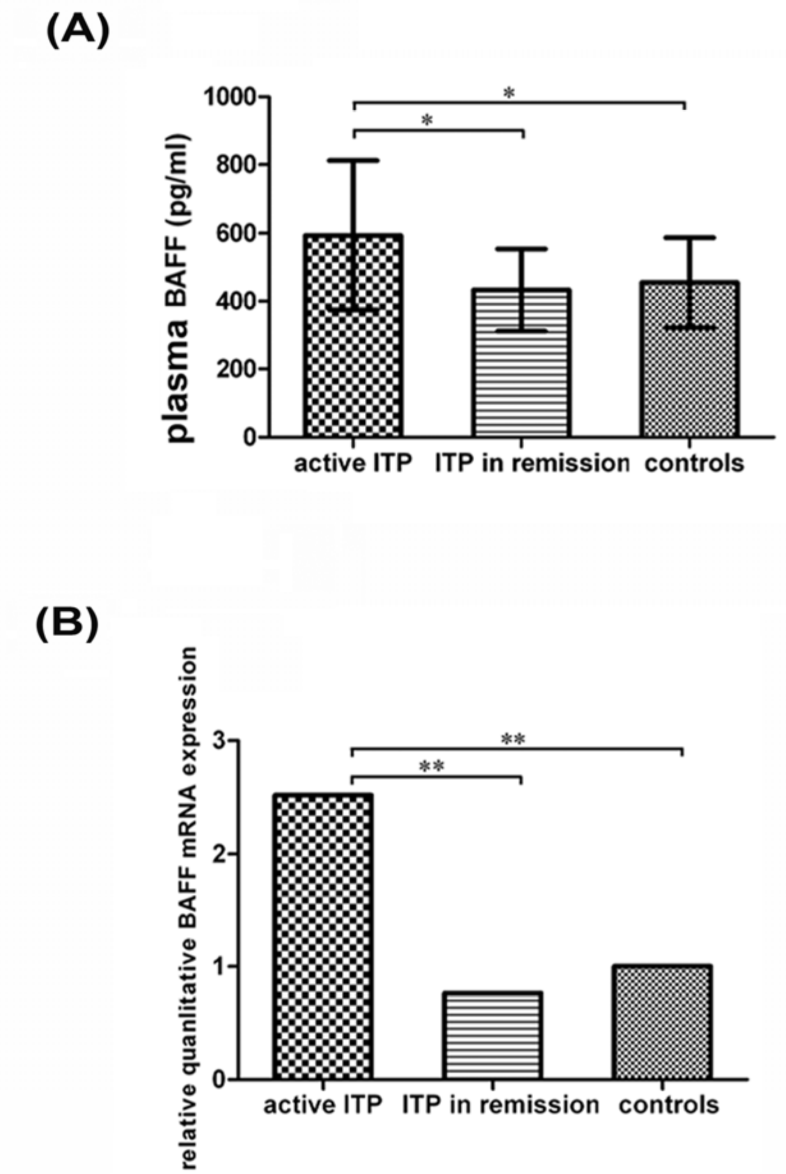

Figure1. The levels of plasma BAFF and BAFF mRNA in ITP patients and controls. (A) Plasma BAFF was elevated in active ITP patients compared to patients in remission (P<0.05) and healthy controls (P<0.05). (B) The ratios of BAFF mRNA in patients with active disease and patients in remission compared to that of healthy

controls are 2.5 ($P<0.01$) and 0.8 ($P>0.05$), respectively. Bars represent SD, * represents $P<0.05$, **represents $P<0.01$.

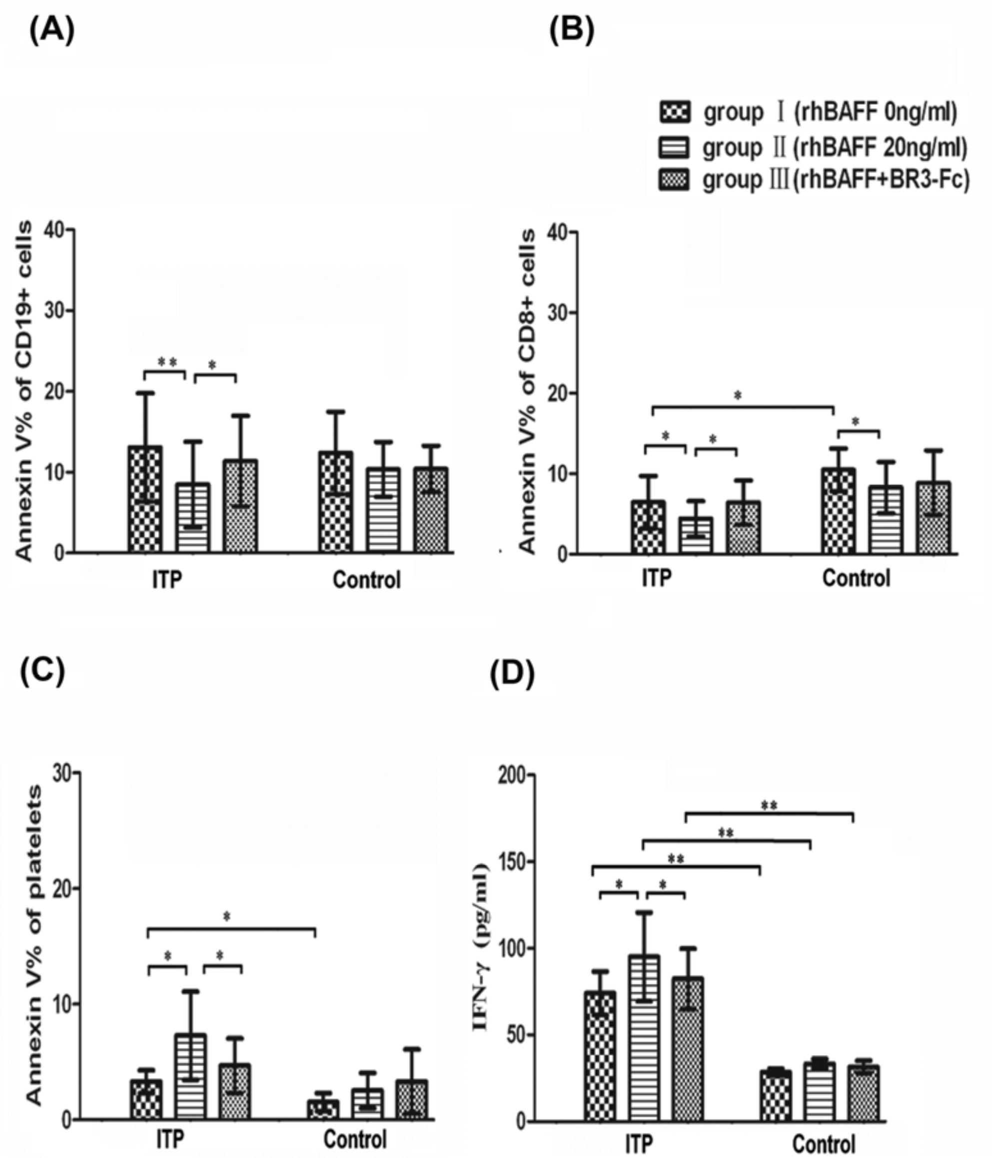

Figure2. Effects of rhBAFF and/or BR3-Fc on apoptosis of CD19$^+$, CD8$^+$ cells, platelets and secretion of IFN-γ in active ITP patients and controls. (A)RhBAFF significantly decreased the annexin V% of CD19$^+$ cells in ITP patients (8.5% vs 13.1%, P<0.01) but not in controls. BR3-Fc corrected the effect of rhBAFF on apoptosis of CD19$^+$ cells in ITP patients (11.4% vs 8.5%, P<0.05). (B) RhBAFF significantly decreased the annexin V% of CD8$^+$ cells in ITP patients and controls. BR3-Fc corrected the effect of rhBAFF on apoptosis of CD8$^+$ cells only in

ITP patients. (C) RhBAFF20 (ng/ml) significantly promoted the annexin V % of platelets only in ITP patients (7.3% vs 3.3%, P<0.05), BR3-Fc corrected the effect of rhBAFF on apoptosis of platelets (4.7% vs 7.3%, P<0.05). When compared to controls, there was significantly increased annexin V % of platelets in groupⅠin ITP patients (3.3% vs 1.3%, P<0.05). (D) RhBAFF20 (ng/ml) significantly promoted the secretion of IFN-γ in ITP patients (95.1pg/ml vs 74.0pg/ml, P<0.05) but not in controls, BR3-Fc corrected the effect of rhBAFF in patients. When compared to controls, there was significantly increased expression of IFN-γ in ITP patients in each group (P<0.01). Bars represents SD, *P<0.05, **P<0.01.

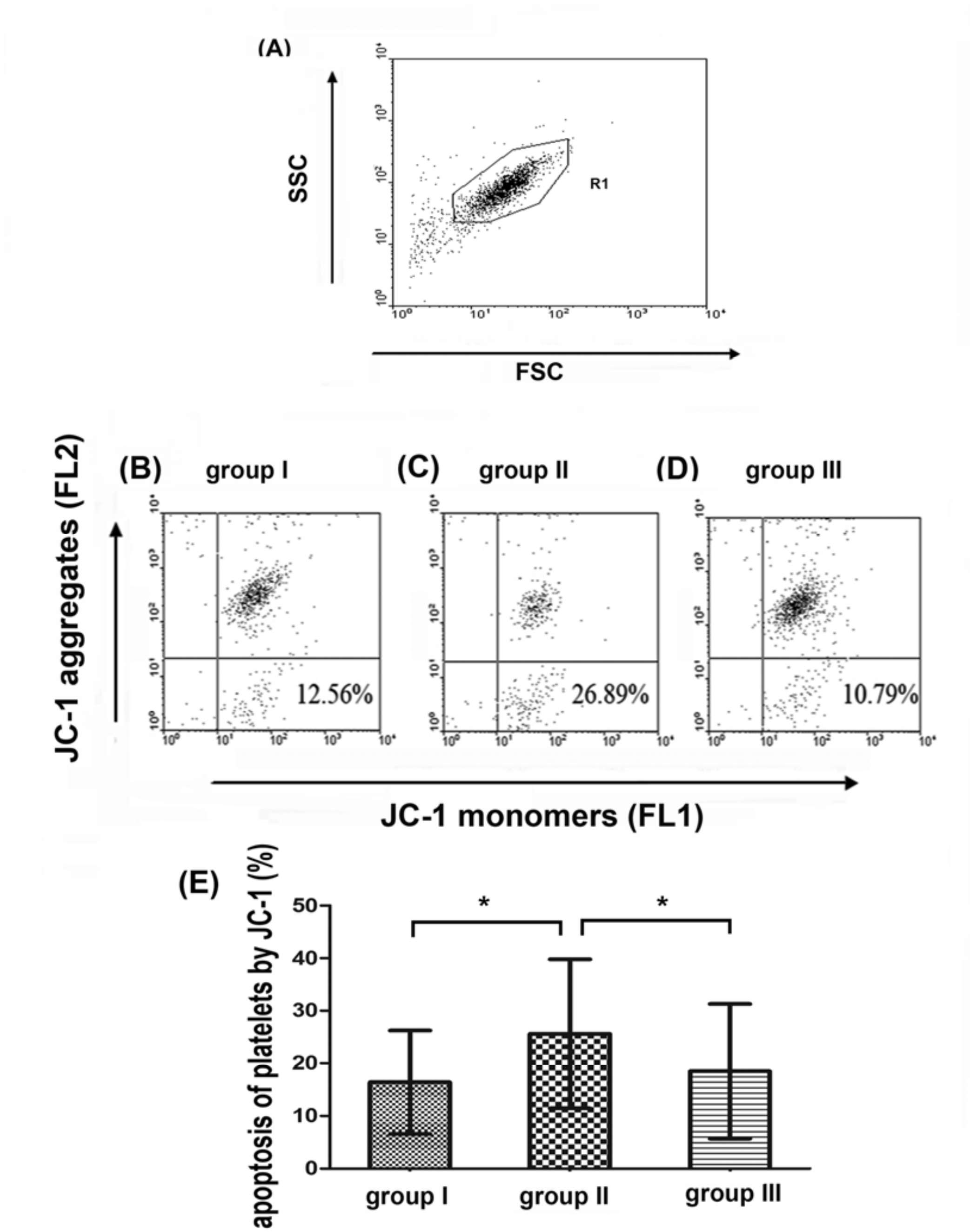

Figure3. **Effects of rhBAFF and/or BR3-Fc on depolarization of mitochondrial transmembrane potential (ΔΨm) in platelets in nine ITP patients.** (A) Platelets were gated by flow cytometry. (B-D) show representative flow cytometric dot plots in different groups. Note that rhBAFF induce ΔΨm depolarization in platelets characterized by transformation of JC-1 dye aggregates to JC-1 monomers, which indicated that rhBAFF promoted early apoptosis of platelets (C). BR3-Fc corrected the effects of rhBAFF on depolarization of ΔΨm in platelets (D). (E) The apoptosis of platelets by JC-1 in different

groups. RhBAFF promoted the apoptosis of platelets and BR3-Fc corrected the effects of rhBAFF. Statistical significance was determined by ANOVA. Bars represent SD, * represents $P < 0.05$.