The effects of BAFF and BAFF-R-Fc fusion protein in immune thrombocytopenia

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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 a clinical benefit in both oncologic and immunologic diseases. In this report, we have detected plasma BAFF and BAFF mRNA expression in immune thrombocytopenia (ITP) patients by enzyme-linked immunosorbent assay (ELISA) and real-time quantitative reverse-transcription polymerase chain reaction (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 interferon γ (INFγ), and interleukin-4 (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, and increased the apoptosis of platelets and the secretion of IFN-γ. BR3-Fc successfully corrected the effects of rhBAFF on lymphocytes, platelets, and cytokines. These findings suggest that BAFF may play a pathogenic role in ITP by promoting the survival of CD19⁺ and CD8⁺ cells, and 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. (Blood. 2009;114: 5362-5367)

Introduction

Immune thrombocytopenia (ITP) is an autoimmune disorder in which the patient’s 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 immunoglobulin G (IgG) antibodies against glycoprotein IIb (GPIIb)/IIa 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 T helper 1 (Th1) bias,³,⁴ the decreased number or defective suppressive function of regulatory T cells,⁵,⁷ and the platelet destruction by cytotoxic T cells (CTLs),⁸,¹⁰ have been described. The cause for these abnormalities remains unknown. Moreover, the treatment regimens for ITP including glucocorticosteroids, 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 B-lymphocyte stimulator, tumor necrosis factor receptor and apoptosis ligand-related leukocyte-expressed ligand 1, tumor necrosis factor homologue that activates apoptosis, nuclear factor κB, and c-Jun NH₂-terminal kinase, and tumor necrosis factor superfamily 13B) belonging to the family of tumor necrosis factor (TNF) ligands is critical for the maintenance of normal B-cell development, homeostasis, and autoreactivity¹¹,¹² and T-cell costimulation.¹³,¹⁵ In addition, BAFF also augments certain Th1-associated inflammatory responses.¹⁶ BAFF binds to 3 receptors: B-cell maturation antigen (tumor necrosis factor receptor superfamily, member 17 [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 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 syndrome (SS),²³ and multiple sclerosis.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 an 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 recombinant human BAFF (rhBAFF) could promote the survival of CD19⁺ and CD8⁺ cells, and increase
the apoptosis of platelets and the secretion of interferon γ (IFNγ), whereas BR3-Fc successfully corrected the effects of rhBAFF on lymphocytes, platelets, and cytokines, suggesting a possible role of BAFF in the pathogenesis of ITP.

**Methods**

**Patients**

Forty-five patients diagnosed with ITP were selected for detection of plasma BAFF and BAFF mRNA. Of these patients, 25 (15 females and 10 males; median age, 41 years; platelets: range, 1-36 x 10^9/L; median, 15 x 10^9/L) were active ITP patients with platelet counts less than 50 x 10^9/L who had not been treated with glucocorticosteroids for at least 1 month before sampling, whereas 20 patients were in remission with normal platelet counts (12 females and 8 males; median age, 37 years; platelets: range, 99-299 x 10^9/L; median, 191 x 10^9/L; supplemental Table 1, available on the Blood website; see the Supplemental Materials link at the top of the online article). 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 an additional 18 ITP patients with active disease (10 females and 8 males; median age, 40 years; platelets: range, 9-49 x 10^9/L; median, 25 x 10^9/L; supplemental Table 2) were selected for detection of apoptosis on CD19+, CD4+, and CD8+ cells and secretion of cytokines; patients 1 to 12 (supplemental Table 2) were also used for detection of apoptosis on platelets. Fifteen healthy controls (9 females and 6 males; median age, 41 years; platelets: range, 157-297 x 10^9/L; median, 218 x 10^9/L) were used for cell culture. Enrollment took place between January 2008 and August 2009 at the Department of Hematology, Qili Hospital, Shandong University. All of the cases met the diagnosis criteria of ITP as previously described. 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 Qili Hospital, Shandong University.

**Preparation of PBMCs and platelets**

PBMCs were isolated from heparinized blood using 1.077 g/mL of ficoll-hypaque (Invitrogen) gradient centrifugation (800g for 20 minutes, 20°C). The isolated PBMCs were washed twice with 0.9% NaCl then resuspended and adjusted to 10^9 PBMCs/mL for cell culture and 10^6 additions. The isolated PBMCs were washed twice with 0.9% NaCl then resuspended and adjusted to 10^6 PBMCs/mL for cell culture. PBMCs were isolated from heparinized blood using 1.077 g/mL of ficoll-hypaque (Invitrogen) gradient centrifugation (800g for 20 minutes, 20°C). The isolated PBMCs were washed twice with 0.9% NaCl then resuspended and adjusted to 10^9 PBMCs/mL for cell culture and 10^6 additions. The isolated PBMCs were washed twice with 0.9% NaCl then resuspended and adjusted to 10^6 PBMCs/mL for cell culture. PBMCs were isolated from heparinized blood using 1.077 g/mL of ficoll-hypaque (Invitrogen) gradient centrifugation (800g for 20 minutes, 20°C). The isolated PBMCs were washed twice with 0.9% NaCl then resuspended and adjusted to 10^9 PBMCs/mL for cell culture and 10^6 additions.

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

The level of plasma BAFF (R&D Systems) was assayed by enzyme-linked immunosorbent assay (ELISA) following the manufacturer’s recommendations. The levels of IFN-γ and interleukin-4 (IL-4) in supernatant of culture were assayed by ELISA (Bender MedSystems). Briefly, 10^6 PBMCs/well with 10^7 autologous platelets/well were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 10 µg/ml phycocerythrin (PHA; Sigma-Aldrich) in 24-well plates (1 mL of final volume) with rhBAFF and BR3-Fc (Genentech) at 37°C with 5% CO2. 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) was used to isolate total RNA. RNA was converted into cDNA using the PrimeScript RT Reagent Kit (Perfect Real Time; Takara) 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) using SYBR Green (Toyobo) 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 minutes) with the following parameters: denaturation at 95°C for 15 seconds; annealing at 60°C for 15 seconds; extension at 72°C for 35 seconds, with temperature transition rates of 20°C/second. The primers for BAFF and β-actin are as follows: BAFF forward: AAGAAGTCGGC- CATTGGACATC; BAFF reverse: TTTCGTTATTTCCATTTGGAGT- TCA; β-actin forward: TTGCAGCAAGATGCAGAA; β-actin reverse: GCCGATCCACAGCGAGACT.

We used the comparative threshold cycle (Ct) method (using arithmetic formulas) for relative quantification of mRNA according to relative expression software tool (REST; Michael). The amplification efficiency between the target (BAFF) and the reference control (β-actin) was compared 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**

PBMCs (10^6/well) with autologous platelets (10^7/well) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum in 24-well plates (1 mL final volume) with rhBAFF (20 ng/mL) or a combination of rhBAFF (20 ng/mL) and BR3-Fc (100 µg/mL) at 37°C with 5% CO2. PBMCs were harvested after 48 hours and incubated with 20 µL of phycoerythrin–cytoxin 5 (Cy5)-conjugated CD19, PE-conjugated CD8 or PE-Cy5-conjugated CD4 (BD Biosciences) for 30 minutes. Cells were washed and incubated with 5 µL of fluorescein isothiocyanate–conjugated annexin V (Invitrogen) for 15 minutes and were analyzed within 1 hour by fluorescence-activated cell sorting.

To investigate the effects of rhBAFF and/or BR3-Fc on the apoptosis of autologous platelets, 10^6 PBMCs/well alone were cultured with rhBAFF or a combination of rhBAFF and BR3-Fc in 24-well plates for 48 hours, and then 10^6 autologous platelets/well were added. After 4 hours, platelets were harvested and incubated with 20 µL of PE-Cy5-conjugated CD41 (BD Biosciences) for 30 minutes, washed, incubated with 5 µL of fluorescein isothiocyanate–conjugated annexin V, and analyzed within 1 hour by fluorescence-activated cell sorting. The assay was to measure annexin V binding to detect membrane phosphatidylserine exposure. Although normal platelet activation also increases annexin V binding, to accurately detect platelet apoptosis, we also measured apoptosis of platelets in an additional 9 ITP patients by mitochondrial membrane potential assay kit with JC-1, which is a marker of mitochondrial activity (Bioytime). In normal undamaged nucleated cells, mitochondrial has a high mitochondrial transmembrane potential (ΔΨm). Breakdown of ΔΨm is characteristic of early apoptosis. As in other cells, ΔΨm 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 reacquires) monomeric form, and show green fluorescence (FL1). Depolarization of ΔΨm was measured by JC-1, which accumulates in mitochondrial matrix, driven by ΔΨm, and expressed as an increase of green to red fluorescence ratio reflecting the transformation of JC-1 aggregates into monomers when mitochondrial membrane becomes depolarized.

**Antiplatelet autoantibody determination**

All plasma samples and cell culture supernatant were stored at −20°C before use. The specific antiplatelet GPIIb/IIIa and/or GPIb/IX autoantibodies were analyzed by modified monoclonal antibody–specific immobilization of platelet antigens, which was carried out as previously described in detail by Hou et al.

**Statistical analysis**

Data were expressed as mean plus or minus SD. Statistical significance was determined by analysis of variance. All tests were performed by SPSS 13.0 system. A P value less than .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 ± 121.4 pg/mL, P < .05) and controls (454.4 ± 132.5 pg/mL, P < .05). No significant difference between patients in remission and healthy controls was found (P > .05).

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

Because rhBAFF significantly promoted the survival of CD8+ T cells that could destruct platelets by cytotoxic T lymphocyte–mediated platelet lysis, we investigated the effects of rhBAFF 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 with patients in remission (P < .01) and healthy controls (P < .01), respectively. Of all the subjects, there was no significant difference between patients in remission and healthy controls (P > .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 percentage 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 percentage of CD8+ cells was significantly decreased in ITP patients (ITP: 6.5% ± 3.2%, controls: 10.5% ± 2.7%, P < .05). RhBAFF significantly decreased the annexin V percentage 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 percentage on CD8+ cells in ITP patients in group I (rhBAFF 0 ng/mL), group II (rhBAFF 20 ng/mL), and group III (rhBAFF + BR3-Fc) was 6.5% (± 3.2%), 4.4% (± 2.2%), and 6.3% (± 2.9%), respectively. The annexin V percentage on CD8+ cells in controls in group I, group II, and group III was 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 percentage of CD4+ cells in both ITP patients and controls (P > .05). The annexin V percentage of CD4+ cells in ITP patients in group I, group II, and group III was 8.6% (± 4.5%), 5.3% (± 1.8%), and 8.2% (± 3.8%), respectively.
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). 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 (10 μg/mL; P < .05) in ITP patients but not controls, and a combination of BR3-Fc and rhBAFF reduced the level of IFN-γ compared with group rhBAFF (20 ng/mL; P < .05). The mean (± SD) of group I was 74.0 (± 12.5) pg/mL, and it increased to 95.1 (± 25.7) pg/mL in group II, and reduced to 82.4 (± 17.4) pg/mL in group III, similar to that in group I (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, whereas 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 that 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, 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 up-regulated 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 et al34 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 primary SS, and rheumatoid
factors in RA). However, some studies found that BAFF levels did not correlate with autoantibody titers in SLE; BAFF stimulation of B cells may contribute to SLE by mechanisms other than autoantibody production. In our study, no association was found between the levels of BAFF and antiplatelet autoantibodies, and addition of rhBAFF did not promote the production of autoantibodies in vitro. These findings suggested that excessive BAFF may not directly promote the production of antiplatelet autoantibodies but may play a role by mechanisms other 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<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells, and found rhBAFF not only promoted the survival of CD19<sup>+</sup> cells but also promoted the survival of CD8<sup>+</sup> cells in ITP patients. Among 18 ITP patients, 14 of them had a larger decrease in annexin V percentage of CD19<sup>+</sup> and CD8<sup>+</sup> cells in group rhBAFF 20 ng/mL than in group rhBAFF 0 ng/mL. BR3-Fc corrected the effects of rhBAFF on annexin V percentage of CD19<sup>+</sup> and CD8<sup>+</sup> cells in these 14 patients. It is of interest to note that the annexin V percentage of CD8<sup>+</sup> cells in ITP patients was significantly decreased compared with healthy controls; more importantly, rhBAFF remarkably promoted the survival of CD8<sup>+</sup> 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, whereas the apoptosis of platelets did not increase without PBMCs, indicating that BAFF may contribute to thrombocytopenia partially by cell-dependent platelet 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 a larger increase in apoptosis of platelets in group rhBAFF 20 ng/mL than in group rhBAFF 0 ng/mL. In these 9 patients, 6 patients (67%) were negative for antiplatelet GPIIb/IIIa and GPIb/IX autoantibodies. The results confirmed our former finding that increasing the apoptosis of CD19<sup>+</sup> and CD8<sup>+</sup> cells in ITP patients. We also found rhBAFF could increase the production of IFN-γ but had no obvious effect on the secretion of IL-4 in vitro experiments, consistent with earlier studies showing typical Th1-mediated responses and reduced Th2-mediated responses in BAFF transgenic mice.

ITP is a heterogeneous disease; besides humoral immune abnormalities, several T-cell abnormalities have been demonstrated in patients with ITP, including Th1 bias, the inhibition of autologous megakaryocyte apoptosis by CD8<sup>+</sup> T cells, and cell-mediated cytotoxic lysis of platelets by CTLs. Our results indicated that elevated BAFF not only was involved in humoral immune abnormalities by promoting the survival of B cells, but also may 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 that could block the interaction of BAFF with all 3 of its receptors. In this study, we demonstrated that BR3-Fc could significantly promote the apoptosis of CD19<sup>+</sup> cells in ITP and block the BAFF-mediated survival of B cells. In addition to promoting the apoptosis of CD8<sup>+</sup> 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. Recent clinical trials with BAFF blockade have shown clinical benefit in SLE and RA. Treatment of lupus-prone New Zealand black/white F1 mice with both BR3-Fc and TACI-Ig significantly decreased the proteinuria and glomerular damage in these mice. 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<sup>+</sup> 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<sup>+</sup> and CD8<sup>+</sup> cells and inhibiting secretion of IFN-γ. Blockade of BAFF by BR3-Fc is a promising therapeutic approach for ITP, especially those with active disease.

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Authorship

Contribution: X.-j.Z. performed research, analyzed data, and wrote the manuscript; Y.S. contributed vital new reagents, designed and performed research, and wrote the manuscript; J.P. performed research, and wrote the manuscript; Y .S. contributed vital new reagents, designed and performed research, and wrote the manuscript.

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