Studying the Effect of rhBAFF & BAFF-R-Fc Fusion Protein on Lymphocytes & Platelets in Children with ITP

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Abstract: Introduction: B cell activating factor, a member of tumor necrosis factor family, is a crucial homeostatic cytokine for B cells. It has been shown to enhance the expression of $CD19^+$ cells and mediate the maturation of autoreactive B cells. BAFF is elevated in several autoimmune diseases including immune thrombocytopenic purpura (ITP). Increased survival of $CD8^+$ T cells also may promote the apoptosis of platelets through cytotoxic T lymphocyte-mediated platelet lysis. Blockade of BAFF receptor has demonstrated a clinical benefit in immunologic diseases. **Methods** PBMCs and platelets from 15 acute ITP patients and 15 healthy controls were cultured with rhBAFF or a combination of rhBAFF and BR3-Fc and then analyzed by flow cytometry for apoptosis of autologous platelets and/or CD19+, CD8+ and CD4+ cells. **Results** Blockade of BAFF receptor by BR3-Fc significantly increased the apoptosis of $CD19^+$ cells in patients only and decreased the apoptosis of platelets in both patients and controls. Apoptotic $CD8^+$ cells were significantly increased in patients only, following the addition of BR3-Fc. **Conclusion** These findings suggest that blockade of BAFF receptor (BR3-FC) could successfully correct the effects of BAFF by promoting the apoptosis of $CD19^+$ and $CD8^+$ cells and decreasing the apoptosis of platelets. Further research will indicate whether blocking BAFF-BR3 will have a therapeutic applicability in the management of ITP or not.

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1. 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).** Autoantibodies against platelet antigens are considered the diagnostic hallmark of ITP **(2)**. The autoantibodies produced by autoreactive B cells against self-antigens, specifically immunoglobulin G (IgG) antibodies against glycoprotein IIb (GPIIb)/IIIa and/or GPIb/IX, are considered to play a crucial role **(3).** Opsonization by antibody not only accelerates platelet clearance but can also alter platelet function and interfere with platelet production **(4)**. Although in most patients ITP is antibody mediated, the autoantibodies are under the control of T helper cells (Th1) and their cytokines. Abnormal T-cell responses drive the differentiation of autoreactive B-cell clones and autoantibody secretion **(5).** It is well known that decreased apoptosis of activated T lymphocytes play an important role in the pathogenesis of ITP **(6).** It was suggested that CD8+ cytotoxic T cells might be involved in the pathogenesis of ITP through cell-mediated destruction of platelets **(7)**, and through suppression of megakaryocyte apoptosis leading to impaired platelet production **(8).**

B-cell activating factor (BAFF) was reported to belong to the tumor necrosis factor (TNF) family, and is thought to be critical for the maintenance of normal B-cell development, homeostasis, and autoreactivity **(9)** and T-cell costimulation **(10,11).** In addition, BAFF also augments certain Th1-associated inflammatory responses **(12).** BAFF is the natural ligand of three unusual tumor necrosis factor receptors termed BCMA, TACI and BR3/BAFF-R **(13,14).** 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 **(15).** Signaling through BR3/BAFF-R and BCMA stimulates B lymphocytes to undergo proliferation and to counter their self destruction by apoptosis. Furthermore, BAFF binding to BR3 on T cells has been shown to costimulate T-cell proliferation both *in vitro* and *in vivo* **(11).**

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 **(16).** In addition, elevated BAFF plasma level was observed in many patients with autoimmune diseases such as rheumatoid arthritis (RA) **(17),** systemic lupus erythematosus (SLE) **(18),** Sjo¨gren syndrome (SS) **(19),** and multiple sclerosis **(20).** Inhibition of BAFF

signaling is a potentially therapeutic option for treatment of B cell–mediated autoimmune conditions.

The aim of the present study is to investigate the effect of rhBAFF and BR3-FC fusion protein on PBMNCs and platelets from ITP patients as well as from normal controls, in a trial to elucidate the role of BAFF in the pathogenesis of ITP.

2. Patients and Methods:

Study population:

Fifteen newly diagnosed cases of acute childhood ITP (disease duration from 0-3 months) were selected from the pediatric wards of New Children's Cairo University Hospital during the time period from Sept 2011 to January 2012. They were diagnosed according to The British Committee for Standards in Hematology **(21)** and included 6 females and 9 males with a median age, 6 years and platelet count range; 9-30 $X10^9$/L with a median count, 18 X 10^9/L. Fifteen healthy children matched for age and sex were included in the study as a control group and included 7 females and 8 males, with a median age 6 years and platelet count range; 160-430 X 10^9/L, with a median count; 200 x 10^9/L. The control subjects were healthy children who presented to Kasr Al-Ainy hospital laboratory for routine checkup. Written informed consents were obtained from the parents of participating children before enrollment.

Exclusion criteria included the following:

Splenomegaly; lymphadenopathy; abnormalities of CBC other than isolated thrombocytopenia with or without mild microcytic hypochromic anemia due to bleeding; abnormal bone marrow picture other than increased and /or defective megakaryocytes; evidence of SLE; recent immunization; drug intake (heparin, sulphonamides, alcohol, quinine) and features of congenital syndromes e.g. absent radii, skeletal anomalies, ocular anomalies or hearing loss.

Preparation of PBMCs:

PBMCs were isolated from 2 ml sample of heparinized blood using 1.077 g/mL of Ficoll-Hypaque (Invitrogen, USA) by density gradient centrifugation method (2000 rpm for 20 minutes, 20°C) as described by Al-Janadi *et al.,* 1993 **(22)**. The isolated PBMCs were then washed twice using sterile phosphate buffered saline then resuspended in 1ml RPMI 1640 and adjusted to 10^6 PBMCs/mL for cell culture.

Preparation of autologous platelets:

Autologous platelets were separated from 5–10 ml sample of heparinized blood, at day 1, by centrifugation at 600rpm for 10 minutes at 20°C. The platelet-rich plasma was separated and acid citrate dextrose (ACD) was added at a ratio one ACD to 9 PRP then recentrifuged at 2500 rpm for 15 minutes and the platelet pellet was washed once using a prepared solution consisting of 0.5 ml ACD and 3 ml PBS and then resuspended in 1 ml RPMI **(23)**.

The effect of rhBAFF and/or BR3-Fc on apoptosis of PBMCs (CD 19+, CD 4+ and CD 8+ cells) by flow cytometry:

PBMCs with autologous platelets were cultured at a ratio 1:10 in RPMI 1640 medium, supplemented with 10% FBS and penicillin-streptomycin 10000 IU/mL, in culture tubes with rhBAFF (R&D systems, USA) alone or with a combination of rhBAFF and BR3-Fc (R&D systems, USA) at 37°C for 24 hrs. Culture tubes were prepared as follows:

Tube 1: contained PBMCs with autologous platelets cultured with 2 µl of rhBAFF, in RPMI medium supplemented with 100 µl FBS, giving a total volume of 1 ml in the culture tube.

Tube 2: contained PBMCs with autologous platelets cultured with 2 µl of rhBAFF and 10 µl of BR3-Fc, in RPMI medium supplemented with 100 µl FBS, giving a total volume of 1 ml in the culture tube. PBMCs were harvested after 24 hrs. For each tube to be analyzed; 3 tubes were prepared, where 100 µL of PBMCs were incubated with either: 20 µL of phycoerythrin (PE)-cyanin 5 (Cy5)-conjugated CD19, 20 µL of PE conjugated CD8 or 20 µL of PE-Cy 5-conjugated CD4 (Beckman Coulter Company, USA) for 30 minutes. Cells were then washed with PBS and incubated with 5 µL of fluorescein isothiocyanate-conjugated annexin 5 (Beckman Coulter Company, USA) for 15 mins. Cells were analyzed within 1 hr by fluorescence- activated cell sorting using Beckman Coulter (Coulter Epics XL).

The effect of rhBAFF and/or BR3-Fc on apoptosis of autologous platelets by flow cytometry:

PBMCs alone were cultured in RPMI 1640 medium supplemented with 10% FBS and penicillin-streptomycin 10000 IU/mL , in culture tubes with rhBAFF alone or with a combination of rhBAFF and BR3-Fc at 37 °C for 24 hrs. By the second day, autologous platelets were separated as described previously and incubated with the cultured PBMCs for 2 hrs. Culture tubes were prepared as follows:

Tube 3: contained PBMCs with autologous platelets cultured with 2 µl of rhBAFF, in RPMI medium supplemented with 100 µl FBS, giving a total volume of 1 ml in the culture tube.

Tube 4: contained PBMCs with autologous platelets cultured with 2 µl of rhBAFF and 10 µl of BR3-Fc, in RPMI medium supplemented with 100 µl FBS, giving a total volume of 1 ml in the culture tube.

Two hrs after addition of platelets, platelets were harvested. For each tube to be analyzed, 1 tube was prepared where 100 µL of platelets were incubated with 20 µL of PE-Cy5-conjugated CD41 (Beckman Coulter Company) for 30 mins. Cells were then washed with PBS and incubated with 5 µL of

fluorescein isothiocyanate-conjugated annexin 5 for 15 mins. Cells were analyzed within 1 hr by fluorescence activated cell sorting. Flow cytometry was adjusted with linear amplification of the forward and side scatter signals. Correlated data of 10,000 events per sample were acquired using dual expression with a live gate applied on CD8+/annexin+ve, CD4+/annexin+ve, CD19+/annexin+ve and CD41+/annexin+ve cells. Two-parameter histograms demonstrating cell staining were created and quadrant statistics were placed on the basis of cell staining.

Interpretation of results:

Results were expressed as a percentage of annexin V (apoptotic) cells with labelled monoclonal antibodies.

Statistical analysis

Descriptive data were expressed as mean plus or minus SD. Paired t-test was used in comparison of means for paired samples while the student's t-test was used in comparison of means for 2 independent groups (cases and controls). Statistical significance was determined by analysis of variance. All tests were performed by SPSS 13.0 system. A *P* value less than 0.05 was considered statistically significant.

3. Results:

The differences in the mean percent expression of annexin V positive T, B lymphocytes and platelets following incubation with rhBAFF / rhBAFF and BR3-Fc between ITP patients and normal healthy controls were statistically evaluated ***(Table 1)***.

Comparison between the percent expression of annexin V positive T, B lymphocytes and platelets before and after addition of BR3-Fc in both ITP patients and control group are shown in ***Table 2***

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

After culture of PBMCs with rhBAFF, the following findings were detected:

The mean percent expression of annexin V positive CD4+ cells was significantly lower in the patients' group than the control group (*P* value <0.033). Addition of BR3-Fc in both patients and control groups lead to an increase in the annexin V positive CD4+ cells, however this increase was not statistically significant ***(Figure 1).***

The mean percent expression of annexin V positive CD8+ cells was significantly lower in the patients' group than the control group (*P* value <0.001). There was a highly significant statistical increase in annexin V positive CD8+ T cells following addition of BR3-Fc in patients only (*P* <0.001), whereas no statistically significant difference was detected in the control group on adding BR3-Fc ***(Figure 2)***.

The mean percent expression of annexin V positive CD19+ cells was also lower in the patients' group than the control group but this decrease was not statistically significant and expression was increased after adding BR3-Fc, which caused a highly significant increase in annexin V positive CD19+ B cells in patients only (*P* <0.001) ***(Figure 3)***.

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

Apoptosis of platelets on addition of rhBAFF and rhBAFF with BR3-FC in the control group was significantly higher than apoptosis of platelets in patients' group (*P* =0.006 & *P* =0.003) respectively. There was a significant statistical decrease in annexin V positive platelets following the addition of BR3-Fc in both patients and controls, this decrease was highly significant in the patient group (*P* <0.001) and it was significant in the control group (*P* <0.003) as seen in ***(Figure 4)***.

Figure 5 represents the apoptosis of platelets in 2 typical ITP patients where rhBAFF increased apoptosis of platelets in ITP patients while BR3-Fc corrected the effect of rhBAFF.

Table (1): Comparison between ITP patients and controls, regarding apoptosis (annexin V expression) on lymphocyte subsets and platelets

	Patients (n= 15)	**Control** (n= 15)	***P***	
CD4 rhBAFF	9.27 ± 3.77	11.85 ± 2.26	0.033	**S**
CD4 rhBAFF+BR-FC3	9.68 ± 4.13	13.03 ± 3.45	0.023	**S**
CD8 rhBAFF	8.06 ± 3.38	20.78 ± 10.2	<0.001	**HS**
CD8 rhBAFF+BR-FC3	11.24 ± 3.54	20.5 ± 9.04	0.002	**S**
CD19 rhBAFF	12.83 ± 4.13	16.96 ± 13.23	0.266	**NS**
CD19 rhBAFF+BR-FC3	16.25 ± 5.73	12.58 ± 11.76	0.290	**NS**
CD41 rhBAFF	11.96 ± 4.33	17.83 ± 6.26	0.006	**S**
CD41 rhBAFF+BR-FC3	8.69 ± 3.9	13.98 ± 4.95	0.003	**S**

*Statistical analysis was done using student t test

Table (2): Comparison between ITP patients and controls regarding apoptotic (annexin V) expression of lymphocyte subsets and platelets, before and after adding BR3-Fc

	CD4 rhBAFF	CD4 rhBAFF+BR3-Fc	*P*	
Patient (n=15)	9.27 ± 3.77	9.68 ± 4.13	0.389	**NS**
Control (n=15)	11.85 ± 2.26	13.03 ± 3.45	0.126	**NS**
	CD8 rhBAFF	**CD8 rhBAFF+BR3-Fc**	***P***	
Patient (n=15)	8.06 ± 3.38	11.24 ± 3.54	<0.001	**HS**
Control (n=15)	20.78 ± 10.2	20.5 ± 9.04	0.809	**NS**
	CD19 rhBAFF	**CD19 rhBAFF+BR3-Fc**	***P***	
Patient (n=15)	12.83 ± 4.13	16.25 ± 5.73	<0.001	**HS**
Control (n=15)	16.96 ± 13.23	12.58 ± 11.76	0.168	**NS**
	CD41 rhBAFF	**CD41 rhBAFF+BR3-Fc**	***P***	
Patient (n=15)	11.96 ± 4.33	8.69 ± 3.9	<0.001	**HS**
Control (n=15)	17.83 ± 6.26	13.98 ± 4.95	0.003	**S**

*Statistical analysis was done using paired t test

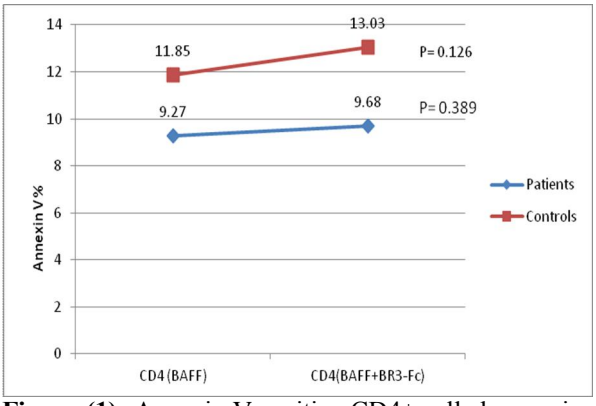


Figure (1): Annexin V positive CD4+ cell changes in patients and controls

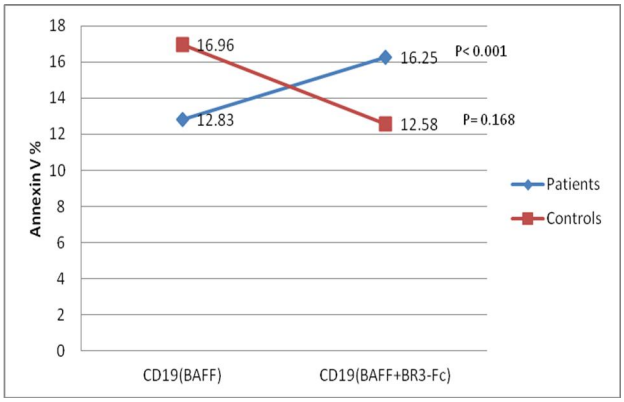


Figure (3): Annexin V positive CD19+ cell changes in patients and controls

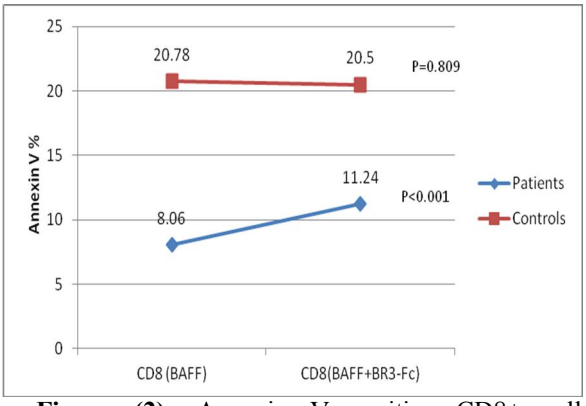


Figure (2): Annexin V positive CD8+ cell changes in patients and controls

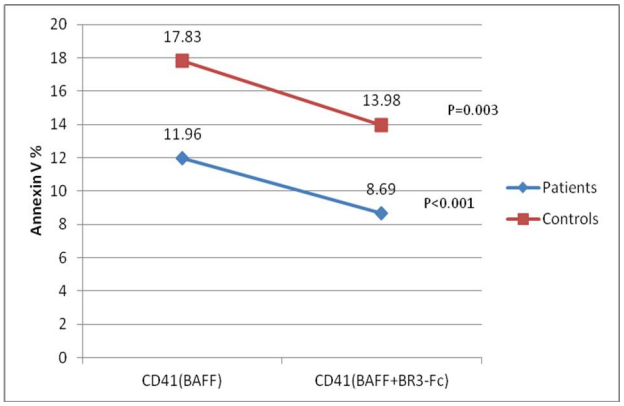
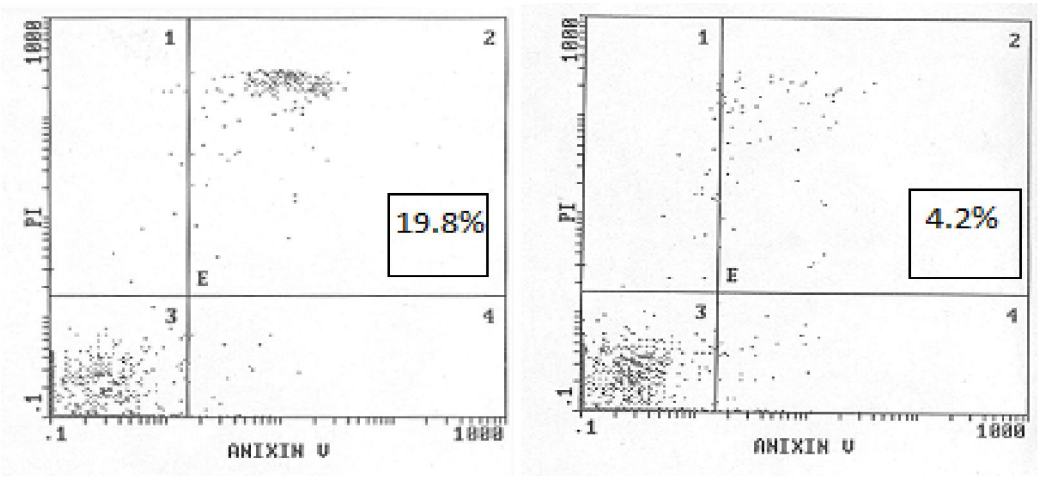


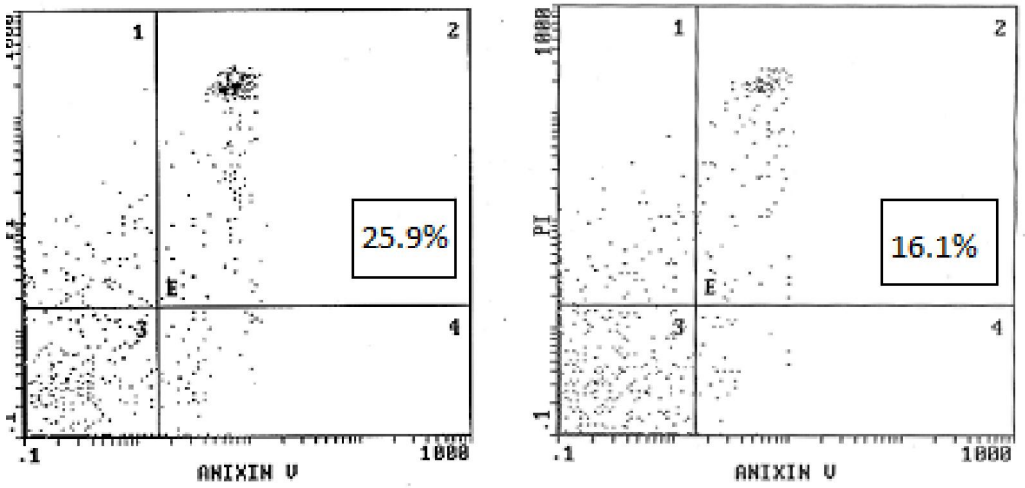
Figure (4): Platelet changes in patients and controls

Patient A :



Effect of rhBAFF alone **Effect of both rhBAFF+BR3-Fc**

Patient B :



Effect of rhBAFF alone **Effect of both rhBAFF+BR3-Fc**

Figure (5): Flow-cytometric dot plots representing the effects of rhBAFF and BR3-Fc on apoptosis of platelets in 2 ITP patients **(A & B)** where rhBAFF promoted the apoptosis of platelets and BR3-Fc corrected the effects of rhBAFF.

4.Discussion:

ITP is an autoimmune disease manifested by immune-mediated platelet destruction and suppression of platelet production. Although several abnormalities involving the cellular mechanisms of immune modulation have been identified, development of autoantibodies against platelet glycoproteins remains central in the pathogenesis of ITP **(4).**

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 cells and mediate the maturation of autoreactive B cells **(24).**

BAFF exerts its role through binding to its receptors. BAFF-R, a specific receptor for BAFF, is expressed on a wide range of B cell subsets and is the predominant receptor that mediates BAFF-dependent B cell signaling and plays critical roles in controlling peripheral B cell survival **(25).** Signaling through BR3/BAFF-R stimulates B lymphocytes to undergo proliferation and to counter their self destruction by apoptosis. Furthermore, BAFF binding to BR3 on T cells has been shown to costimulate T-cell proliferation both *in vitro* and *in vivo* **(11).**

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 **(26)**.

In a study by ***Zhu et al., 2009*****,** the level of plasma BAFF and BAFF mRNA in ITP patients with active disease was found to be significantly higher than that in patients in remission and controls **(26)**. It was also noted that high-dose dexamethasone (HD-DXM) has shown its clinical efficacy in ITP patients **(27)**.

Wang et al., 2010 investigated the change of BAFF and regulatory T-cells before and after high-dose dexamethasone therapy and assessed the effect of BAFF on Treg cells in ITP. They concluded that HD-DXM might play a role in ITP treatment by down-regulating BAFF expression and up-regulating Treg cells number **(28)**.

In the present study, we tried to elucidate the effect of rhBAFF on PBMNCs and platelets, and the effect of blocking BAFF-R on the survival of these cells. Fifteen patients diagnosed as acute ITP were selected for this study in addition to fifteen healthy subjects as a control group. Basic investigations for diagnosis of ITP were performed including complete blood picture and bone marrow aspiration. Culture of isolated lymphocytes and platelets was then done with rhBAFF or rhBAFF and BR3-Fc, and analysis of apoptotic cells was performed using dual expression flowcytometry.

After culture of PBMCs with rhBAFF, we found that the mean percent expression of annexin V positive CD4+ and CD8+ cells were significantly lower in the patients' group than the control group (*P* value = 0.033, *P* value =<0.001) respectively. The mean percent expression of annexin V positive CD19+ cells was also lower in the patients' group than the control group but this decrease was non significant statistically. In a study by ***ZHU et al.*****, 2009** they demonstrated that rhBAFF significantly decreased the annexin V percentage of CD19+ cells in ITP patients but not in controls, while it significantly decreased the annexin V percentage of CD8+cells in both ITP patients and controls (ITP: 6.5%±3.2%, controls: 10.5%±2.7%, *P*<0.05). On the other hand they found no significant effect of rhBAFF on annexin V percentage of CD4+cells in both ITP patients and controls (*P* <0 .05) **(26)**.

In this study, we demonstrated that the decrease in the apoptotic expression of different lymphocyte subsets from ITP cases was corrected after adding BR3-Fc, which caused an increase in annexin V positive CD4+ cells, however this increase was not statistically significant**.** On the other hand, there was a highly significant statistical increase in annexin V positive CD8+ T cells and CD19+ B cells following addition of BR3-Fc (P<0.001 in both). Our results were in agreement with ***ZHU et al., 2009*** study where BR3-FC corrected the decrease in annexin V percentage of CD19+ cells caused by rhBAFF in 14 ITP patients. It also corrected the effect of rhBAFF on apoptosis of CD8+cells only in ITP patients, while they stated that there was no significant effect for rhBAFF+ BR3-FC on annexin V percentage of CD4+ cells in both ITP patients and controls **(26)**.

Concerning the annexin V percentage of CD41+ platelets, we detected a significant statistical decrease in annexin V positive platelets following the addition of BR3-Fc in both patients' group and control group, this decrease was highly significant in the patient's group and was moderately significant in the control group. ***ZHU and his co-workers*****, 2009** demonstrated that rhBAFF significantly increased apoptosis of platelets in ITP patients but not in controls. Addition of BR3-Fc corrected the effect of rhBAFF on apoptosis of platelets. Further study showed that the apoptosis of platelets increased in ITP patients when autologous platelets were incubated with PBMNCs from the same patient after rhBAFF was added whereas the apoptosis of platelets did not increase without PBMNCs, indicating that BAFF may contribute to thrombocytopenia partially by cell-dependent platelet destruction in ITP **(26)**.

Our study 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 promoting the apoptosis of CD8+ cells, BR3-Fc also inhibited the apoptosis of platelets. BAFF blockade could result in B-cell reduction in animal models **(29,30)**. Recent clinical trials with BAFF blockade have shown clinical benefit in SLE and RA **(31,32)**. These findings offer further support to the contention that blockade of BAFF signaling may be of therapeutic benefit in a variety of autoimmune diseases.

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 apoptosis of platelets. Blockade of BAFF by BR3-Fc could be a promising therapeutic approach for ITP.

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