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B-Cell Activating Factor (BAFF) is elevated in Chronic Granulomatous Disease

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Abstract

Chronic Granulomatous Disease (CGD) is an inherited defect in superoxide production leading to life-threatening infections, granulomas, and, possibly, abnormal immunoglobulin concentrations. We investigated whether factors controlling antibody production, such as B-cell activating factor (BAFF), were altered in CGD. CGD subjects had significantly increased mean (2.3-fold, $p < 0.0001$) plasma concentrations of BAFF compared to healthy donors. Patients on IFN- γ treatment had significantly higher BAFF concentrations compared with CGD patients not taking IFN- γ (1.6-fold, $p < 0.005$). Leukocytes from CGD subjects produced normal amounts of BAFF in response to IFN- γ or G-CSF *in vitro*. Expression of BAFF-R and TACI were significantly reduced on CGD B cells. Elevated BAFF in CGD correlated with CRP ($R = 0.44$), ESR ($R = 0.49$), and IgM ($R = 0.47$) and increased rapidly in healthy subjects following intravenous endotoxin administration. These findings suggest that elevated BAFF in CGD subjects and healthy donors is a consequence of acute and chronic inflammation.

Keywords

Chronic Granulomatous Disease; B-cell activating factor; inflammation

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Conflict of interest statement

The authors declare no conflict of interest.

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1. Introduction¹

Chronic Granulomatous Disease (CGD) is an inherited immunodeficiency secondary to mutations affecting the NADPH oxidase enzyme resulting in life-threatening infections and exuberant inflammation with granulomas that can obstruct vital structures such as the genitourinary and gastrointestinal tracts (1). Previous studies have demonstrated dysfunctional microbicidal activities and increased proinflammatory mediator production by CGD polymorphonuclear cells (2–4). Additionally, T lymphocytes from CGD patients produce more TH₁₇ cytokines (5) which may be one mechanism contributing to autoinflammatory complications reported in CGD patients such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (6, 7). The initial clinical descriptions of CGD noted recurrent infections in young boys marked by hypergammaglobulinemia and plasmacytosis (8). Knockout mice deficient in p47^{phox}, a model of CGD, demonstrated higher specific antibody titers than wild-type mice in response to bacterial challenge (9). Moreover, CGD patients exhibit increased antibody levels to some commensal flora compared with healthy donors suggesting increased exposure of CGD subjects to these antigens (10). CGD patients exhibit a markedly altered B cell compartment characterized by a diminished absolute number and percentage of CD27+ memory B cells, with no significant difference in absolute numbers of naïve B cells (11). A recent study confirmed the paucity of memory B cells circulating in CGD patients but further demonstrated that patients mount normal antibody responses to vaccination antigens such as influenza (12). CGD patients also have an increased incidence of a range of autoimmune diseases, some of which have been characterized within the NIH cohort (6) in addition to other case reports (13).

Given reported abnormalities in the abundance of memory B cells, we studied circulating factors known to stimulate B cells in these patients. Numerous cytokines influence immunoglobulin production, including IL-21 and several TNF-superfamily members such as B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) that augment B-cell receptor (BCR)-triggered activation (14–16). Neutrophils, monocytes, and T cells produce BAFF upon stimulation with pro-inflammatory cytokines such as IFN- or G-CSF (17, 18). Elevated levels of IL-21, BAFF, and APRIL have been found in autoimmune diseases such as SLE, certain primary immunodeficiencies, chronic inflammatory states, and some cancers (19–23). Increased levels of these cytokines, and BAFF in particular, have been found to correlate with immunoglobulin levels, autoantibody titers, and exacerbated symptoms in autoimmune and neoplastic diseases (24, 25).

2. Materials and methods

2.1. Subjects

All human blood samples used in this study were collected after informed consent under NIH IRB-approved protocols 91-I-162, 93-I-0119, 05-I-0213, 06-I-0160, 07-I-0075, 08-EI-0169, 99-CC-0168, and 2010-I-0029.

A total of 117 CGD patients (27 females and 90 males, mean age of 25 ± 12 years, mean $\pm$ standard deviation) and 80 healthy donors (42 ± 12 years) were studied for measurements of B cell activating cytokines BAFF, APRIL, and IL-21. Patient characteristics are described in Table 1.

2.2. Serology

Plasma BAFF and APRIL levels were measured by ELISA (Quantikine, R&D Systems, Minneapolis, MN) and IL-21 with the READY-SET-Go ELISA (eBioscience, La Jolla, CA) on frozen plasma samples that had been stored at -80°C or liquid nitrogen. C reactive protein (CRP) was measured using fixed time nephelometry. Plasma and serum immunoglobulin G

(IgG), immunoglobulin M (IgM), and immunoglobulin A were measured by nephelometry using the Siemens Dimension Vista System (Siemens Healthcare Diagnostics Inc., Newark, DE, USA). Immunoglobulin E (IgE) was analyzed by chemiluminescence assay on a Siemens Immulite 2500 (26).

2.3. Cell isolation and stimulation

Fresh peripheral blood mononuclear cells (PBMC) were isolated from citrated blood using Ficoll Plus (GE Healthcare BioSciences Corp., Piscataway, NJ) and suspended in phenol red-free RPMI 1640 supplemented with glutamine and 10 mM HEPES pH 7.2 (Gibco, Grand Island, NY). PBMC were cultured at a concentration of 2×10^6 cells/ml and stimulated with IFN- γ (200 U/ml, eBioscience) for 72 h. Fresh polymorphonuclear cells (PMN) were isolated as previously described, with purity exceeding 95%, and cultured at a concentration of 5×10^6 cells/ml in the presence of G-CSF (1,000 U/ml, eBioscience) for 24 h (27). Culture supernatants were then collected and frozen at -80°C for later measurement of secreted BAFF.

2.4. Flow Cytometry

Fresh cells were blocked for 15 minutes at 4°C with Fc receptor block (eBioscience) prior to staining with antibodies and appropriate isotype controls: anti-CD19, anti-CD267 (TACI), anti-CD268 (BAFF-R), and anti-B cell maturation antigen (BCMA; eBioscience). Data were acquired using a FACScalibur (BD Biosciences, San Jose, CA) and analyzed with FlowJo software (Treestar, Inc., San Carlos, CA)(28).

2.5. Endotoxin challenge studies

BAFF was measured from thawed plasma stored for 15 years at -80°C from previously reported healthy subjects challenged with intravenous RE-2 endotoxin after informed consent (NIH IRB approved protocol 91-I-162)(29). WBC and temperature of the subjects receiving endotoxin were reported previously (28).

2.5. Statistical analysis

Statistical testing was performed using GraphPad Prism (Version 5, GraphPad Software, Inc., La Jolla, CA). Levels of significance were defined as $p = 0.05$ (*), $p < 0.01$ (**), and $p < 0.0001$ (****) using the Mann-Whitney test (to compare continuous outcomes between groups), Wilcoxon signed-rank test (for paired data), and Spearman correlation coefficient (association between continuous outcomes) where specified.

3. Results

3.1. Elevated Plasma BAFF, but not IL-21 or APRIL in CGD

Plasma BAFF was significantly ($p < 0.0001$) elevated in CGD patients (mean $\pm$ SD of 1476 ± 1125 pg/ml, $n=117$) vs. healthy donors (650 ± 196 pg/ml, $n=80$; Fig 1, A). APRIL was not significantly increased in CGD (CGD: 10431 ± 20453 pg/ml; $n=58$ vs normal: 2706 ± 2651 pg/ml; $n=10$; Fig 1, B). IL-21 was not increased in CGD subjects (CGD: 127 ± 76 pg/ml; $n=58$ vs normal: 126 ± 44 pg/ml; $n=10$ ($p = 0.65$)).

3.2. BAFF among CGD subgroups

We next tested whether BAFF concentrations in CGD patient plasma were associated with genotype, gender, IFN- γ treatment, prophylactic antimicrobials, steroid administration, active infection, gastrointestinal disease, or autoimmune disease. CGD females had significantly higher levels (21% increase) of plasma BAFF compared to CGD males (Table 1). There was no significant difference in plasma BAFF in healthy men and women. Among

age- and sex-matched normal subjects and CGD (part of the data shown in Fig 1), plasma BAFF remained significantly elevated. Subjects receiving IFN- γ had significantly higher (62%) BAFF levels compared to CGD patients not receiving IFN- γ (Table 1). Other subgroups were not significantly different (Table 1).

3.3. Receptor expression for BAFF is reduced on CGD B cells

CD19⁺ B cells from normal and CGD PBMC from clinically stable patients were sorted with magnetic beads and analyzed by FACS to determine the relative expression levels of the three receptors for BAFF: BAFF-R (CD268), TACI (CD267), and BCMA. Significantly lower levels of BAFF-R and TACI were detected on CGD CD19⁺ B cells (Fig 2, A and B). BCMA was not detected on purified CD19 cells from patients or normal subjects (data not shown). Using median forward scatter as a marker of B cell size, we did not detect a statistically significant difference between normal (312 ± 49 units; $n=12$) and CGD B cells (300 ± 31 units; $n=6$), nor did we detect a correlation between plasma BAFF and expression of BAFF-R ($R=-0.03$; $p = 1.00$; $n = 6$) or TACI ($R=-0.26$; $p = 0.66$; $n = 6$).

3.4. Systemic BAFF correlates with markers of inflammation and age in CGD subjects

Correlations between BAFF, immunoglobulin levels, and peripheral B cell counts have been reported (24, 30–33). Therefore, we assessed whether there was a correlation of elevated plasma BAFF and immunoglobulins, acute phase reactants, or autoantibodies in our CGD patients. Plasma BAFF positively correlated with the acute phase reactants CRP and ESR as well as IgM levels in 29 subjects for whom measurements were available (Fig 3, A–C). BAFF negatively correlated with age (Fig 3, D) as reported previously in healthy newborns and small children (34). Plasma BAFF did not correlate significantly with residual neutrophil superoxide production, a predictor of survival in CGD neutrophils (35) or plasma APRIL levels (Fig 3, E and F). Moreover, plasma BAFF did not significantly correlate with peripheral B cell numbers ($R=-0.25$; $p = 0.17$; $n = 32$). We did not detect a significant correlation of plasma BAFF with other immunoglobulin classes (IgA, IgG, and IgE) or autoantibodies (ANA, anti-ENA, and RF). Sixteen of 29 subjects (55%) had a positive ANA, which did not significantly correlate with plasma BAFF.

3.4. BAFF secretion by CGD PBMC and PMN is normal

Neutrophil production of two chemoattractants, IL-8 and LTB₄, is increased in CGD (2, 36). We therefore tested whether CGD PMN or PBMC produced more BAFF in response to IFN- γ or G-CSF, two stimuli shown to promote BAFF secretion *in vitro* (17, 18). Upon stimulation of PMN or PBMC with IFN- γ , BAFF secretion did not differ significantly between normal (86 ± 35 pg/ml, $n=7$) and CGD (76 ± 30 pg/ml, $n=5$) PBMC, nor was there a significant difference between IFN- γ -treated normal (155 ± 72 pg/ml, $n=7$) and CGD (110 ± 34 pg/ml, $n=6$) PMN. Similarly, there was no significant difference between normal and CGD PMN stimulated with G-CSF (normal 237 ± 117 pg/ml, $n=7$ and CGD PMN 196 ± 91 pg/ml, $n=6$). Supernatants collected from unstimulated cells failed to produce any BAFF as detected by the ELISA. None of the CGD subjects were receiving IFN- γ or G-CSF at the time their cells were collected.

3.5. Plasma BAFF is independent of acute infection

The correlation between plasma BAFF and IgM suggested that BAFF might be responsive to acute infection. Although plasma BAFF was elevated in 16 patients who were culture proven or clinically suspected of having infection, the difference was not significant between clinically infected and uninfected CGD patients (Table 1). For 8 subjects, we had paired samples from the period when they had culture positive lung biopsies (*Aspergillus* ($n=4$), *Burkholderia* ($n=3$), and *Nocardia* ($n=1$)) and from when they were clinically stable.

There was no significant difference between BAFF levels when these patients were clinically stable (1385 ± 589 pg/ml) or actively infected (1044 ± 476 pg/ml). Two other CGD patients who expired from *Aspergillus* pneumonias within 30 days of obtaining the samples had high plasma BAFF (8528 and 3891 pg/ml) but paired samples were not available from those patients; no other premorbid samples were available for study.

3.6. Effect of endotoxin challenge on BAFF production in healthy volunteers

BAFF was measured in stored plasma samples from three healthy subjects (mean age: 28 years) who had undergone intravenous endotoxin challenge (3 ng *E. coli* LPS/kg) as reported previously (29). By 6 h post-endotoxin administration, plasma BAFF levels increased at least 3-fold and by 24 h increased up to 5-fold (Fig 4). The kinetics of the BAFF response was distinct from the transient increases in temperature and leukocytes reported in the original study and reproduced for three subjects in Fig 4 (29).

4. Discussion

This study is the first demonstration of elevated plasma BAFF in CGD. Such findings have been reported in other diseases, such as SLE, where plasma BAFF is high and positively associated with CRP (37). Our work demonstrates that CGD subjects can have documented plasma BAFF on par with other autoimmune diseases like SLE (37), but not as high as reported for multiple myeloma (38). Though plasma APRIL levels trended higher in CGD patients, this difference did not achieve statistical significance. Moreover, IL-21 did not appear to be different between normal and CGD patients. We show a positive correlation between plasma BAFF and acute phase reactants and IgM in our CGD population. However, unlike studies on autoimmune disease and gammopathies like multiple myeloma, we did not find an association between BAFF and other immunoglobulin classes (IgG, IgA, and IgE) or autoimmunity (21, 25). Patients on IFN- γ prophylaxis to prevent infection (39) had significantly increased plasma BAFF (Table 1) compared with the elevated levels in other CGD patients. CGD B cells had diminished expression for two BAFF receptors, BAFF-R and TACI, akin to other diseases such as Sjögren's syndrome in which elevated plasma BAFF was found (40). Similar B-cell size between normal and CGD, as determined by FACS, suggests that the difference in MFI is due to something other than membrane area. Whether or not diminished receptor expression decreases cellular responsiveness to BAFF or causes the observed accumulation of BAFF in the circulation remains unclear.

In addition to finding a relationship between BAFF and inflammation in CGD patients, we found that BAFF concentrations in the circulation of healthy individuals increase in response to intravenous endotoxin. While previous studies have indicated that increased plasma BAFF may accompany chronic inflammatory diseases (41), this is the first report of BAFF alterations *in vivo* in response to bacterial product in otherwise healthy donors. BAFF has been reported to promote TH₁/TH₁₇-mediated inflammation through downstream cellular events (42, 43). Moreover, BAFF triggers the production of pro-inflammatory cytokines through the activation of the NF- κ B pathway (44). Intriguingly, hyperinflammation in CGD patients may also result from aberrant activation of lymphocytes, as suggested by the production of TH₁₇-skewed proinflammatory cytokines (5). Our data suggest that elevated BAFF is a marker of inflammation in CGD and the acute phase response in healthy donors.

BAFF appears to act as an acute phase reactant and frequent inflammation in immunodeficiencies may keep its levels high. It is also possible that altered consumption of BAFF or production by non-circulating cells may regulate BAFF levels. Accordingly, the hypergammaglobulinemia sometimes found in CGD may be a consequence of frequent infection and chronic antigenic stimulation leading to high states of B cell activation *in vivo*.

(45). While we show increased BAFF in clinically stable and infected CGD subjects and in healthy donors following endotoxin administration, it remains unknown whether BAFF triggers the formation autoreactive antibodies. Future studies will be needed to further delineate whether elevated BAFF in CGD contributes to the exaggerated inflammation or predisposition to autoimmune disease.

5. Conclusions

CGD patients have significantly higher levels of plasma BAFF. Among CGD patients, BAFF is higher in those taking IFN- γ , elevated BAFF moderately correlates with acute phase reactants, and inversely correlates with age. Upon stimulation *in vitro*, patient PMN and PBMC produced normal amounts of BAFF, suggesting that increased plasma BAFF is a consequence of inflammation in CGD, decreased consumption due to lower receptor expression levels, or altered production by other cell types.

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ABBREVIATIONS

CGD	Chronic granulomatous disease
BCR	B cell receptor
BAFF	B cell activating factor
TACI	transmembrane activator and CAML-interactor

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Highlights

- Plasma BAFF is significantly increased in CGD patients
- Among CGD patients, plasma BAFF correlates with markers of acute inflammation
- BAFF levels are further elevated among CGD patients taking IFN-
- BAFF levels increase within 6 hours in healthy donors given intravenous endotoxin
- BAFF may be a marker of poorly regulated inflammation in CGD

Figure 1. Circulating BAFF in CGD. (A) Plasma BAFF measured by ELISA in 80 healthy donors and 117 CGD patients. (B) Plasma APRIL measured in 10 healthy donors and 58 CGD patients. P-values determined by Mann Whitney t test.

Figure 2. BAFF receptors in CGD. (A) representative histograms of isotype control (grey), CGD (white), and healthy donor (black) for BAFF-R and TACI expression on sorted CD19+ B cells. (B) median fluorescent intensity (MFI) for BAFF-R and TACI for CGD and healthy subjects. * $p < .05$ by Mann Whitney t test.

Figure 3. BAFF associations in CGD. Correlation of plasma BAFF with (A) C-reactive protein, (B) erythrocyte sedimentation rate, (C) IgM, (D) age, (E) residual neutrophil superoxide production, and (F) APRIL levels. P-values determined by Spearman R .

Figure 4.
Plasma BAFF in three healthy volunteers injected intravenously with *E. coli* endotoxin.

Table 1

Patient characteristics

Category	BAFF mean $\pm$ SD pg/ml (n)	p-value ^a
<i>Genotype</i>		
gp91 ^{phox}	1485 $\pm$ 1290 (68)	0.11 ^b
p22 ^{phox}	1767 $\pm$ 354 (5)	
p47 ^{phox}	1330 $\pm$ 813 (37)	
p67 ^{phox}	2037 $\pm$ 1312 (7)	
<i>Sex</i>		
Male	1410 $\pm$ 1159 (90)	0.05
Female	1708 $\pm$ 1009 (27)	
<i>IFN- treatment^c</i>		
No	1358 $\pm$ 941 (98)	0.004
Yes	2106 $\pm$ 1718 (19)	
<i>Prophylactic antibiotics^d</i>		
No	1608 $\pm$ 1291 (7)	0.77
Yes	1345 $\pm$ 738 (94)	
<i>Prophylactic antifungals^e</i>		
No	1389 $\pm$ 834 (19)	0.73
Yes	1357 $\pm$ 774 (87)	
<i>Steroids^f</i>		
No	1493 $\pm$ 1134 (81)	0.69
Yes	1449 $\pm$ 1135 (36)	
<i>Active infection^g</i>		
No	1363 $\pm$ 781 (101)	0.29
Yes	2214 $\pm$ 2264 (16)	
<i>Gastrointestinal disease^h</i>		
No	1605 $\pm$ 1307 (77)	0.26
Yes	1237 $\pm$ 610 (40)	
<i>Autoimmune disease</i>		
No	1488 $\pm$ 1155 (106)	0.93
Yes	1391 $\pm$ 882 (11)	

^a p-values are based on a Mann-Whitney t test.

^b p-value based on a Kruskal-Wallis test one-way analysis of variance.

^c patients receiving up to 50 μ /m² subcutaneously three times a week

^d receiving antimicrobials for prophylaxis rather than treatment of an acute infection

^e receiving antifungals for prophylaxis rather than treatment of an acute infection

^f patients receiving any dose of prednisone would be under “yes”.

^g determined by culture positivity in 8/16 patients and high clinical suspicion in the remainder (constitutional symptoms, WBC > 11,000/ μ l, temperature > 38.5C) prompting additional treatment with antibiotics

^h patients diagnosed with colitis or fistulas