

Regulation of B-Cell-Activating Factor Expression on the Basophil Membrane of Allergic Patients

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Key Words

Allergic bronchopulmonary aspergillosis · *Aspergillus fumigatus* · B-cell-activating factor · Basophil · Basophil activation test · Fungal sensitization · IgE · Severe asthma · TLR-2

Abstract

Background: To investigate the modulation of B-cell-activating factor (BAFF) expression on the basophil membrane of allergic patients. BAFF is an important regulator of B-cell activation, proliferation and immunoglobulin production, which may play a role in respiratory allergic diseases in promoting the production of IgE by B cells. **Methods:** Peripheral blood samples of 10 patients with allergic rhinitis, 3 with severe asthma and fungal sensitization (SAFS), 3 with allergic bronchopulmonary aspergillosis (ABPA) and 11 healthy controls were assessed regarding BAFF (CD257) expression using the basophil activation test before and after stimulation with IgE and allergens, as well IgE-independent stimuli, like fMLP, lipoteichoic acid from *Staphylococcus aureus* (LTA-SA) and lipopolysaccharide (LPS). **Results:** BAFF membrane expression did not change after IgE and allergen stimulation both in patients and controls, while it was upregulated by *Aspergillus* stimulation, both in sensitized patients and controls. In both patients and controls, BAFF expression was sig-

nificantly upregulated following LTA-SA and β -1,3-glucan exposure (toll-like receptor-2 ligands), but not following LPS stimulation. **Conclusions:** Basophils from allergic and healthy subjects constitutively express membrane BAFF, which is not upregulated by IgE or specific allergens but by TLR-2 ligands (LTA-SA and β -1,3-glucan). *Aspergillus fumigatus* stimulation was able to upregulate BAFF expression on the basophils of sensitized asthmatic patients, but not via IgE-dependent mechanisms, since results did not differ between the patient and control groups. These findings suggest that basophils may contribute to the polyclonal production of IgE commonly observed in patients with SAFS and ABPA.

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Introduction

B-cell-activating factor (BAFF) is an important regulator of B-cell activation, proliferation and immunoglobulin production. It plays an important role in autoimmune diseases [1]. Recent observations indicate that BAFF may also play a role in respiratory allergic diseases [2]. Allergic rhinitis and asthma are characterized by the production of IgE specific for ubiquitous allergens (e.g. pollen, mites and molds); in some patients, an increase in total unspecific IgE may be observed, particularly in patients with

severe asthma combined with fungal sensitization (SAFS) [3] or with allergic bronchopulmonary aspergillosis (ABPA) [4]. B-cell-stimulating factors such as BAFF are thought to play a role in promoting the production of IgE by B cells. It has been shown that allergen-specific B cells are activated and undergo class switch recombination at allergic reaction sites [5, 6], and the release of BAFF in bronchoalveolar lavage fluid has been reported after specific allergen challenge in allergic asthmatics [2]. Basophils are an important component of both immediate allergic reactions and chronic inflammation, and due to their myeloid origin, these cells may be a source of BAFF production. The mechanisms of BAFF expression or release by basophils are only partly known. Chen et al. [7] demonstrated that IgD-binding antigens increased basophil expression of BAFF, and activation of toll-like receptors (TLRs) in basophils from healthy controls induced IgG4 production by B cells, an effect associated with enhanced production of BAFF [8]. These observations also point to a role of basophils in adaptive immune responses, similar to other cells of the innate immune system, including neutrophils, macrophages and monocytes [9, 10]. The aim of this study was to investigate whether BAFF is expressed on the basophil membrane of allergic patients and whether its membrane expression is increased by IgE-dependent stimuli, such as allergen exposure, and/or IgE-independent stimuli, including TLR engagement. Specifically, we were interested to investigate the effect of *Aspergillus* stimulation on BAFF expression on the basophil membrane of patients with SAFS or ABPA, who showed high serum levels of total IgE.

Patients and Methods

Study Design

Peripheral blood samples of 16 allergic rhinitis patients (aged 48 ± 17.1 years: 10 males and 6 females) with or without asthma and 11 healthy controls (aged 51 ± 12.5 years; 7 males and 4 females) were assessed for BAFF (CD257) expression following the basophil activation test (BAT) before and after IgE stimulation with specific allergens (e.g. grass, ragweed and *Aspergillus fumigatus*) and the TLR-4 ligand, lipopolysaccharide (LPS). In the 6 *Aspergillus*-sensitized patients (3 had severe asthma, total IgE: 418 ± 348 kU/l; 3 had ABPA, total IgE: $2,399 \pm 1,265$ kU/l), BAT was also performed after stimulation with the TLR-2 ligands, lipoteichoic acid from *Staphylococcus aureus* (LTA-SA) and β -1,3-glucan from *Euglena gracilis*, as well as fMLP, as IgE-independent stimuli. BAFF mRNA expression was quantified by RT-PCR in 2 patients and 2 controls. All the patients and controls gave their informed consent to participate in the study, which was approved by the local institutional review board. The patients' demographic and clinical characteristics are reported in table 1.

Table 1. Clinical and demographic characteristics of the allergic patients (n = 16) grouped according to allergen sensitization

	Ragweed	Grass	<i>A. fumigatus</i>	Total IgE, kU/l (mean \pm SD)
Gender				
Male	2	4	4	–
Female	3	1	2	–
Age (mean \pm SD)	39.5 \pm 13.5	57 \pm 12.5	60 \pm 15.5	–
Rhinitis	3	2	–	–
Rhinitis + asthma	2	3	–	–
Severe asthma	–	–	3	418 \pm 347.8
ABPA	–	–	3	2,399 \pm 1,265

Basophil Activation Test

BAT was performed according to a previously reported technique. Briefly, endotoxin-free heparinized whole-blood samples were obtained from allergic patients and healthy controls. Cells were challenged with 100 μ l of anti-IgE (10 μ g/ml; clone G7-18; BD Bioscience, USA), allergens (0.2 μ g/ml ragweed, 2.5 μ g/ml grass and 2.5 μ g/ml *A. fumigatus*; ALK Abelló, Italy), fMLP (0.5 μ g/ml; Sigma Aldrich, Italy) or TLR ligands (1 μ g/ml LTA-SA and 2.5 μ g/ml β -1,3-glucan; both from InvivoGen, USA, and 1 μ g/ml LPS from *Escherichia coli*; Sigma Aldrich) for 20 min at 37°C in a water bath (optimal stimulation times were assessed in previous experiments). The optimal stimulation concentrations of the anti-IgE, allergens, fMLP and TLR ligands were established in preliminary experiments. As a negative control, Tyrode solution (Sigma Aldrich) with 20 μ M HEPES and 7.5% NaHCO₃, pH 7.4, was used to assess the spontaneous expression of the different markers. The reactions were terminated by chilling the cells on ice.

Immunophenotyping and Flow-Cytometric Analyses

Basophils were stained with 5 μ l of anti-human Fc ϵ RI α -Pacific Blue (BioLegend, USA), 10 μ l of anti-human CD63-FITC (clone H5C6; BD Biosciences), 10 μ l of anti-human CD203c-APC (clone NP4D6; BioLegend) and 10 μ l of monoclonal anti-human CD257-PE (BioLegend) for 20 min on ice. Flow-cytometric analyses were performed on a FACSCanto II flow cytometer (BD Immunocytometry Systems, USA). Side scatter and anti-IgE+/CD203c+ staining were applied to gate out at least 500 basophils. BAFF positivity was set on basophils with a fluorescence intensity that was above the level of the 99th percentile of a FMO (Fluorescence Minus One) sample.

Basophil Purification

Basophils were isolated using immunomagnetic methods (MACS basophil isolation kit II; Miltenyi). Briefly, PBMCs from 2 healthy subjects and 2 allergic patients were resuspended in PBS with 0.5% BSA and 2 mM EDTA, and were incubated with a basophil biotin antibody cocktail after FcR blocking. The basophils that were assessed by flow cytometry (using Fc ϵ RI α -Pacific Blue and CD203c-APC) accounted for more than 95% of the total cells.

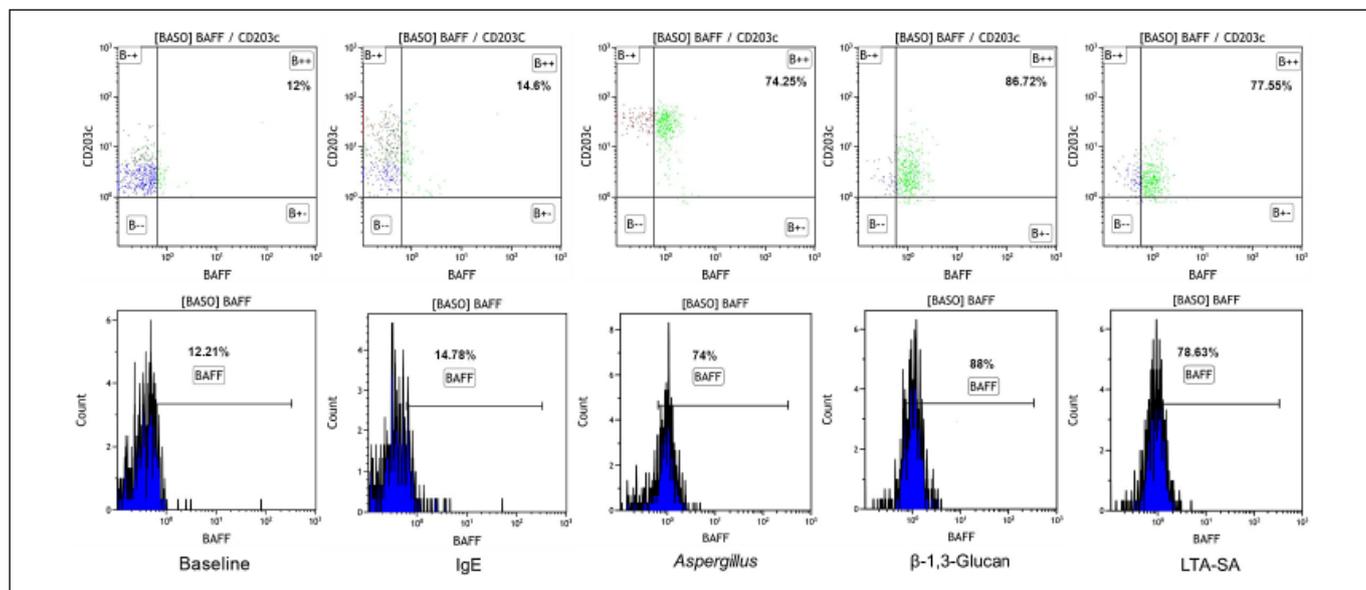


Fig. 1. The effect of basophil stimulation on membrane BAFF expression in a patient. Basophils were gated as IgE⁺/CD203c⁺. *A. fumigatus*, LTA-SA and β -1,3-glucan induce significant upregulation of BAFF on the basophil membrane.

Real-Time PCR Analysis for BAFF mRNA Expression

Total RNA from purified basophils was extracted after lysis in TRIzol reagent (Invitrogen Life Technologies). RNA samples were reverse transcribed at 42°C with a reverse transcription system (Promega, USA) according to the manufacturer's instructions, and the subsequent cDNA was used for real-time PCR. Quantitative real-time PCR assays were performed using primers that specifically amplified BAFF and G6PD (Hs00198106_m1 and Hs00166169_m1, respectively; purchased from Applied Biosystems). RT-PCR was performed with a BioRad iQ iCycler (BioRad, USA) and analyzed with the gene expression analysis for iCycler iQ real-time PCR detection system software (BioRad). BAFF and G6PD primer amplification efficiencies were validated in a preliminary experiment. For PCR analysis, each sample was run in duplicate in separate tubes. The results were normalized to a housekeeping gene (G6PD), as shown by Vandesompele et al. [11].

Statistics

All the variables are reported as means \pm SD. Comparisons between patients and controls were performed using Student's t test for paired data. All statistical analyses were performed using GraphPad, Prism 4.

Results

BAFF was constitutively expressed on the basophil membrane (fig. 1), with no difference between the allergic patients ($19 \pm 15.8\%$ basophils) and controls ($19.3 \pm 10.5\%$ basophils).

BAFF mRNA Expression

BAFF mRNA expression on purified nonstimulated basophils did not differ between patients and controls, confirming the flow-cytometric finding (data not shown).

IgE-Dependent Stimuli

BAFF membrane expression did not change after IgE stimulation in both patients and controls (from 19 ± 15.8 to 25.3 ± 17.4 in patients, and from 19.3 ± 10.5 to 19.6 ± 9.6 in controls), whereas CD63 was upregulated as expected. BAFF membrane expression did not significantly change following grass and ragweed stimulation (from 13.8 ± 17.8 to 23.5 ± 16.8 in patients, and from 17.7 ± 9.6 to 23.6 ± 9.4 in controls), whereas CD63 expression was significantly upregulated (from 0.33 ± 0.12 to 33.4 ± 0.28 , $p < 0.0001$) on the allergic patients' basophil membranes as expected.

Aspergillus Stimulation

BAFF expression was significantly upregulated on the basophil membrane of patients and controls following *A. fumigatus* stimulation (from 27.7 ± 11.7 to 57 ± 17.1 in patients, $p = 0.0012$, and from 27.8 ± 6.2 to 58.3 ± 7.6 , $p = 0.0002$, in controls; fig. 2a).

IgE-Independent Stimuli

In both patients and controls, BAFF expression was significantly upregulated following LTA-SA (from 26.4 ± 12.9 to 63 ± 10.6 in patients, $p = 0.0063$, and from 20 ± 8.7

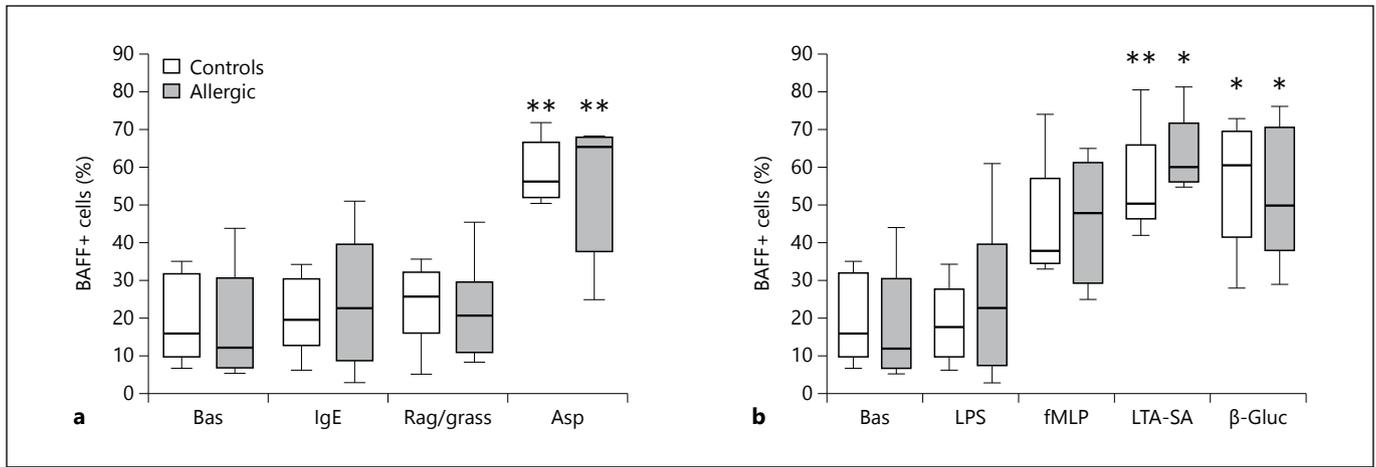


Fig. 2. The percentage of BAFF-positive cells at baseline, after IgE and allergen stimulation (**a**), and after non-IgE-related (**b**) stimuli in controls and allergic subjects. Rag = Ragweed; grass = grass pollen; Asp = *A. fumigatus*. Bas = baseline; β -Gluc = β -1,3-glucan. * $p < 0.05$, ** $p < 0.01$.

to 55.2 ± 16.8 in controls, $p = 0.023$) and β -1,3-glucan exposure (from 26.4 ± 12.9 to 53.4 ± 18 in patients, $p = 0.036$, and from 20 ± 8.7 to 55.5 ± 19.7 in controls, $p = 0.021$). Following fMLP exposure, BAFF expression did not significantly increase in patients (from 26.4 ± 12.9 to 45.6 ± 16.5 , $p = 0.15$); however, it increased almost significantly in controls (from 20 ± 8.7 to 45.7 ± 19 , $p = 0.06$). Additionally, LPS stimulation did not change BAFF expression in either patients or controls (fig. 2b).

Discussion

Our results show that basophils of allergic and healthy subjects constitutively expressed membrane BAFF and that its expression was not upregulated by IgE or specific allergens, though it was upregulated by TLR-2 ligands (LTA-SA and β -1,3-glucan). *A. fumigatus* stimulation was able to upregulate BAFF expression on the sensitized asthmatic patients' basophils, which showed also increased CD203c and CD63 expression, as expected. The mechanism of BAFF expression could not be explained by an IgE-dependent pathway, as the same level of BAFF upregulation was also observed in nonallergic controls, who did not show any changes in CD203c and CD63 expression. Considering the fast stimulation time (20 min), it is conceivable that BAFF is contained in a vesicle storage compartment and that TLR-2 stimulation promotes the specific granules to be fused to the plasma membrane and expressed. Similarly to the independent CD203c and CD63 membrane expression, which is regulated by the

activation/degranulation of cells [12], our results suggest that BAFF expression might follow a specific pathway of activation that is independent of degranulation/CD63 expression and CD203c/CD11b expression.

TLRs recognize fungal pathogen motifs, including *Aspergillus* motifs, and both TLR-2 and TLR-4 have been shown to recognize *A. fumigatus* hyphae [13]. In our experiments, LPS, a TLR-4 ligand, was not able to upregulate membrane BAFF, indicating that membrane BAFF expression may be upregulated by *A. fumigatus* following TLR-2 engagement. Watanabe et al. [8] demonstrated that LPS induced the release of BAFF, suggesting a possible difference between surface expression and the release of BAFF after TLR-4 stimulation.

Following fMLP exposure, another IgE-independent stimulus, BAFF expression increased, even if the increase was not significant, due to the broad distribution of the response, suggesting that there is heterogeneity in the studied population, where certain individuals have a propensity to express more BAFF following stimulation with fMLP. The reasons for this heterogeneous response are not clear.

We did not measure BAFF release from basophils because our observation was limited to its membrane expression, but BAFF membrane expression may be an important step toward its release. Membrane BAFF cleavage by inflammatory cytokines has been reported to be a mechanism of BAFF release by neutrophils [14].

Basophils are cells of myeloid origin, similar to neutrophils, and migrate from the blood compartment to inflamed tissue, where they act as both effector inflamma-

tory cells and cells that are involved in pathways linking innate to adaptive immunity. Specifically, basophils may help to regulate the adaptive immune response because they are a known early source of IL-4 and other cytokines associated with Th2 responses [15]. Our finding that BAFF is constitutively expressed on the basophil membrane of allergic patients may have implications for the activation of immunoglobulin production and class switching recombination. Other cells, such as epithelial cells, may produce BAFF and participate in the promotion of local IgE production, as reported for chronic rhinosinusitis with nasal polyps [16], where sinonasal tissue BAFF mRNA expression was significantly correlated with CD20 expression [17].

The upregulation of BAFF expression on the basophil membrane that we observed following *Aspergillus* stimulation may be related to the huge amount of polyclonal IgE production that is commonly observed in patients with SAFS and to an even higher degree in ABPA patients, the airways of whom are colonized by *Aspergillus* [3, 4].

In conclusion, our results demonstrate that basophils constitutively express BAFF on their membranes, both in allergic and in healthy subjects, and are able to upregulate its expression through non-IgE-mediated stimulation. These findings suggest that basophils, like other cells of innate immunity, help to establish a link between innate and adaptive immune responses, specifically following TLR stimulation.

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Disclosure Statement

The authors have no conflict of interest to declare.

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