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

Monica Boita\textsuperscript{a} Enrico Heffler\textsuperscript{a} Stefano Pizzimenti\textsuperscript{a} Alberto Raie\textsuperscript{a} Elona Saraci\textsuperscript{b} Paola Omedè\textsuperscript{b} Claudia Bussolino\textsuperscript{a} Caterina Bucca\textsuperscript{a} Giovanni Rolla\textsuperscript{a}

\textsuperscript{a}Division of Allergy and Immunology, Department of Medical Science, Azienda Ospedaliera Ordine Mauriziano Umberto I, and \textsuperscript{b}Division of Hematology, Azienda Ospedaliera Città della Salute e della Scienza di Torino, University of Turin, Turin, Italy

\textbf{Key Words}
Allergic bronchopulmonary aspergillosis · 	extit{Aspergillus fumigatus} · B-cell-activating factor · Basophil · Basophil activation test · Fungal sensitization · IgE · Severe asthma · TLR-2

\textbf{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.\textit{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, lipotheichoic acid from \textit{Staphylococcus aureus} (LTA-SA) and lipopolysaccharide (LPS). \textit{Results}: BAFF membrane expression did not change after IgE and allergen stimulation both in patients and controls, while it was upregulated by \textit{Aspergillus} stimulation, both in sensitized patients and controls. In both patients and controls, BAFF expression was significantly upregulated following LTA-SA and $\beta$-1,3-glucan exposure (toll-like receptor-2 ligands), but not following LPS stimulation. \textit{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 $\beta$-1,3-glucan). \textit{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.

\textbf{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...
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 ± 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.

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

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

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Bafil Expression on Basophils

BAFF Expression on Basophils

**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 μl of anti-IgE (10 μg/ml; clone G7-18; BD Bioscience, USA), allergens (0.2 μg/ml ragweed, 2.5 μg/ml grass and 2.5 μg/ml A. fumigatus; ALK Abelló, Italy), fMLP (0.5 μg/ml; Sigma Aldrich, Italy) or TLR ligands (1 μg/ml LTA-SA and 2.5 μg/ml β-1,3-glucan; both from InvivoGen, USA, and 1 μg/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 μM 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 μl of anti-human FcεRα-Pacific Blue (BioLegend, USA), 10 μl of anti-human CD63-FITC (clone H5C6; BD Biosciences), 10 μl of anti-human CD203c-APC (clone NP4D6; BioLegend) and 10 μl 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 FcεRα-Pacific Blue and CD203c-APC) accounted for more than 95% of the total cells.
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 ± 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 ± 15.8% basophils) and controls (19.3 ± 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 to 58 ± 10.6 in controls, p = 0.0004).
to 55.2 ± 16.8 in controls, p = 0.023) and β-1,3-glucan ex-
posure (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 sig-
nificantly in controls (from 20 ± 8.7 to 45.7 ± 19, p = 0.06).
Additionally, LPS stimulation did not change BAFF ex-
pression 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 in-
creased 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 ex-
pression. Considering the fast stimulation time (20 min), 
it is conceivable that BAFF is contained in a vesicle stor-
age 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 ex-
pression and CD203c/CD11b expression.

TLRs recognize fungal pathogen motifs, including As-
pergillus motifs, and both TLR-2 and TLR-4 have been 
shown to recognize A. fumigatus hyphae [13]. In our ex-
periments, LPS, a TLR-4 ligand, was not able to upregu-
late 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 pos-
sible difference between surface expression and the re-
lease 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 pro-
pensity 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 be-
cause our observation was limited to its membrane ex-
pression, but BAFF membrane expression may be an im-
portant 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 neutro-
phils, and migrate from the blood compartment to in-
flamed 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.

**Acknowledgment**

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**Disclosure Statement**

The authors have no conflict of interest to declare.

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