Aberrant expression of BAFF in T cells of systemic lupus erythematosus, which is recapitulated by a human T cell line, Loucy

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Abstract

B cell-activating factor of the tumor necrosis factor (TNF) family, or BAFF, is mainly produced in monocytes and dendritic cells, and indispensable for proliferation, differentiation and survival of B cells. BAFF is a type II membrane-bound protein and the extracellular C-terminal fragment is released from the cells as soluble BAFF (sBAFF), which binds to specific receptors on B cells. Accumulating evidence suggests that BAFF plays an important role in the pathogenesis of autoimmune diseases, such as systemic lupus erythematosus (SLE). In this study, we developed a sensitive sandwich ELISA system to quantify the amount of sBAFF using our own mAb. Treatment of peripheral T cells of SLE patients with an anti-CD3 antibody triggered robust expression of BAFF and subsequent release of sBAFF from the cells. On the other hand, the stimulus induced only marginal elevation of sBAFF from normal T cells. These data indicate that BAFF is expressed in T cells upon stimulation at least under pathological conditions. Expression of BAFF was also largely induced in a human T cell line, Loucy (American Type Tissue Collection CRL-2629), in response to several stimuli, while other T cell lines so far examined produced the cytokine almost constitutively. These data suggest that Loucy recapitulates some of the characteristics of SLE T cells. Investigation of molecular and cellular mechanisms of production of BAFF in Loucy demonstrated that expression of BAFF was regulated through a signal transduction pathway which involves c-jun NH2-terminal kinase and p38, and that shedding of BAFF was catalyzed by a membrane-bound protease, furin.

Introduction

B cell-activating factor (BAFF), which is also called TNF ligand superfamily, member 13b, BLys, TALL-1, zTNF-4 and THANK, is a cytokine which belongs to the tumor necrosis factor (TNF) ligand family. It is known that BAFF plays a crucial role in proliferation, differentiation and survival of B cells (1–8). BAFF is a type II membrane-bound protein of 285 amino acid residues, and a C-terminal fragment (from Ala134 to Leu285) is released from cells possibly as a trimer (3). Released soluble BAFF (sBAFF) binds to its receptors; that is, TACI, BCMA and BAFF-R (9–15), and activates several factors including c-jun NH2-terminal kinase (JNK) and nuclear factor-κB (2, 11, 13, 14, 16).

It is noteworthy that transgenic mice that over-express BAFF in lymphoid cells developed hyperplasia of mature B cells (9, 17, 18), which is one of the typical symptoms of systemic lupus erythematosus (SLE). In addition, it has been reported that the serum level of BAFF was elevated in patients afflicted with SLE (19–23). Taken together, these data strongly suggest that abnormal expression of BAFF results in aberrant production of autoreactive IgG from B cells, which may underlie the development of SLE. Therefore, elucidation of the mechanism of the aberrant BAFF production is important not only to understand the mechanism of pathogenesis of SLE but also to develop a therapeutic strategy to treat the disease.

Several groups reported that BAFF is mainly expressed in PBMCs and dendritic cells and not expressed in T cells (1, 4, 5). However, another group insisted that T cells expressed...
Expression of BAFF in human T cells

BAFF (3). In addition, recent study has shown that BAFF is expressed in T cells infiltrating salivary glands from patients of Sjögren’s syndrome (24). These data imply that BAFF is expressed in T cells under certain pathological conditions. Therefore, it is intriguing to investigate whether or not BAFF is also expressed in SLE T cells.

In the present study, we revealed that the expression of BAFF was largely induced in peripheral T cells prepared from SLE patients upon stimulation via TCRs. In an attempt to investigate the mechanism of expression of BAFF in T cells in more detail, we searched for a human T cell line that showed inducible expression of BAFF and found that a human T cell line named Loucy met the criteria.

Methods

Antibodies, recombinant proteins and chemicals
A rabbit polyclonal anti-BAFF antibody, recombinant human sBAFF and recombinant human APRIL (soluble form) were purchased from Chemicon International (Temecula, CA, USA). A mouse monoclonal anti-human CD3 antibody, HRP-conjugated streptavidin and recombinant human IFN-γ were purchased from BD PharMingen (San Diego, CA, USA). Biotinylated goat anti-rabbit IgG, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), ionomycin, SP600125, SB203580 and total RNA Extracting Kit are products of Sigma (St Louis, MO, USA). A mouse monoclonal anti-β-actin antibody and an FITC-conjugated mouse monoclonal anti-human CD3 antibody were purchased from R&D Systems (Minneapolis, MN, USA). Block Ace and decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone were purchased from Dainippon Pharmaceuticals (Osaka, Japan) and ALEXIS Biochemicals (Lausen, Switzerland), respectively. ReverTra Dash and PCR primers for IFN-γ, IL-2 and TNF-α were purchased from Toyobo (Osaka, Japan). A primer set and TaqMan Probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as well as TaqMan Universal PCR Master Mix were purchased from Applied Biosystems (Foster City, CA, USA).

Recombinant DNA
A cDNA fragment (945 bp) encoding human BAFF was amplified by reverse transcription (RT)-PCR using total RNA prepared from normal peripheral blood lymphocytes as a template and the following oligonucleotides as primers: 5′-agggctcaacctccaaagttcaag (P1) and 5′-cttagaaacagacaggaagggag (P2). The resultant fragment was inserted into pTarget (Promega, Madison, WI, USA) to construct pTarget/FL-BAFF. Analysis of the nucleotide sequence showed that the cDNA fragment encoded entire BAFF rather than delta BAFF, a splice variant of BAFF which lacks 57 nucleotides within exon 3 (25).

Preparation of anti-BAFF mAbs
Mice were immunized with a keyhole limpet hemocyanin-conjugated synthetic oligopeptide which corresponds to the N-terminal region of sBAFF (i.e., NH2-AVQGPEEVTQDC-COOH). Splenocytes of the mice were fused with P3-X63-Ag8-U1 according to the standard procedure (26), and hybridoma cells producing anti-BAFF antibodies were selected by ELISA using the oligopeptide as an antigen. A hybridoma clone (4H4) obtained by limiting dilution was injected into mice intraperitoneally with pristane. IgG fraction was purified from the ascites and biotinylated for sandwich ELISA. In brief, the antibody was mixed with biotin (long arm) N-hydroxysuccinimide ester (Vector Laboratories, Burlingame, CA, USA) in sodium bicarbonate buffer (pH 9.0), stirred at room temperature for 2 h and then extensively dialyzed against PBS at 4°C.

ELISA
A rabbit anti-BAFF antibody (1 μg ml⁻¹ in PBS) as a capture antibody was dispensed into a 96-well Immuno Plate (NUNC, Rochester, NY, USA) and kept overnight at 4°C. The wells were then blocked with Block Ace overnight at 4°C. Culture media of T cells were centrifuged at 800 × g for 7 min at 4°C, and the supernatants were serially diluted with the growth medium. The capture antibody was exposed to the supernatants overnight at 37°C. sBAFF was detected by sequential incubation at room temperature with biotinylated anti-BAFF mAb (4H4, 2 μg ml⁻¹) as a detection antibody for 2 h, then with HRP-conjugated streptavidin (1 μg ml⁻¹) for 1 h and finally with TMB one solution (Clontech, Palo Alto, CA, USA) for 10 min. The reaction was stopped by adding 1 N HCl, and optical densities at 450 nm were measured with a spectrophotometer (Dainippon Pharmaceuticals, Tokyo, Japan). The concentration of BAFF was estimated from a calibration curve prepared with recombinant sBAFF.

Cell culture
Molt-4, Jurkat, CCRF-CEM and Loucy [American Type Tissue Collection (ATCC) CRL-2629] were purchased from ATCC (Manassas, VA, USA). THP-1 and HLCL-1 (JCRB 0041) were purchased from Japanese Collection of Research Bioresources (Osaka, Japan). In order to prepare peripheral T cells, venous blood samples were collected from SLE patients and healthy individuals under informed consent. T cells were enriched by mixing whole blood with RosetteSep T Cell Enrichment Cocktail (StemCell Technologies, Vancouver, Canada). The purity of the preparation was >90%, which was estimated by FACs using an FITC-conjugated anti-CD3 antibody. Peripheral T cells and the cell lines were cultured at 2.5 × 10⁵ ml⁻¹ in RPMI 1640 (ATCC) supplemented with 10% FCS and maintained in a humidified incubator (7% CO₂) at 37°C. For induction of BAFF, the cells were cultured in a six-well plate (cell lines) or a 24-well plate (peripheral T cells) previously coated with a mouse monoclonal anti-human CD3 antibody (10 μg ml⁻¹) at 4°C overnight (27). Alternatively, the cells were stimulated with TPA (50 ng ml⁻¹) and ionomycin (0.5 or 1 μg ml⁻¹), which mimics stimulation via TCR. Viabilities of the cells were determined by MTT assay (28).

PCR analyses
Total RNAs were extracted from cells using total RNA Extracting Kit, and 20 ng each of RNA were subjected to RT using ReverTra Dash as a reverse transcriptase. PCR was then carried out for BAFF, IFN-γ, IL-2 and TNF-α using AdvanTaq DNA polymerase (Clontech). P1 and P2 (described above) were used as a primer set for BAFF. The reaction conditions were as follows: 90 s at 94°C for denaturation and 90 s at 66°C.
for annealing and primer extension (35 cycles). The resultant DNA fragments were analyzed with agarose gel electrophoresis. For real-time PCR analysis, one-forth each of the cDNA products was used as a template. The following oligonucleotides were used as primers for BAFF: 5'-cgcgggactgaatacttgg and 5'-cacgcatttctgtgtcttgta. TaqMan Probe (5'-ccaccagtctcaggagaagccaat) was synthesized by Applied Biosystems. Reactions were carried out with TaqMan Universal PCR Master Mix, and fluorescence was detected by ABI PRISM 7700 (Applied Biosystems). The reaction conditions were as follows: 15 s at 95°C for denaturation and 60 s at 60°C for annealing and primer extension (40 cycles). The mRNA level for BAFF underwent dual normalization against those of GAPDH and then unstimulated cells at time 0 h.

Western blot analysis
Cells were harvested, washed once with PBS and disrupted in 0.2 ml of M-Per (Pierce Biotechnology, Rockford, IL, USA). Thirty micrograms of proteins/lane were separated through a 12.5% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane filter (Millipore, Billerica, MA, USA). The membrane was blocked with Block Ace at 4°C overnight and then incubated with an anti-BAFF polyclonal antibody [1 µg ml⁻¹ in 20 mM Tris–HCl (pH 7.6), 0.05% Tween 20 (TTBS)] at room temperature for 2 h. After extensive washing with TTBS, the membrane was incubated with biotinylated goat anti-rabbit IgG (0.1 µg ml⁻¹) and HRP-conjugated streptavidin (0.2 µg ml⁻¹) at room temperature for 1 h. The membrane was extensively washed with TTBS, followed by incubation with ECL (Amersham Biosciences, Piscataway, NJ, USA). Immunoreactivities were visualized by exposing Instant B&W film (Fuji Photo Film, Tokyo, Japan) to the membrane.

Results

Establishment of ELISA system
We prepared a hybridoma clone (4H4) that produces a mAb (subclass IgG1) against sBAFF, and successfully developed a sensitive sandwich ELISA system to measure the concentration of sBAFF. Optical densities at 450 nm almost linearly increased in proportion to the concentration of sBAFF at the range of 0.4–100 ng ml⁻¹ (Fig. 1, closed circles). The ELISA system was highly selective to BAFF and did not cross-react with APRIL [TNF ligand superfamily, member 13 (29)], which has a homology with BAFF (Fig. 1, open circles).

Induction of BAFF in peripheral T cells
Peripheral T cells were prepared from SLE patients and normal subjects and cultured for 96 h. The amounts of sBAFF in the culture media were measured by ELISA. SLE and normal T cells produced 8.1 ± 1.5 and 1.6 ± 1.4 ng ml⁻¹, respectively, of sBAFF in vitro in the absence of stimulation. It may be noteworthy that SLE T cells produced higher amount of sBAFF than normal T cells under basal conditions (Fig. 2a).

Inducible expression of BAFF in Loucy
Peripheral T cells were then stimulated with an anti-CD3 antibody, a stimulus which triggers signal transduction within T cells via TCR. Proliferation of the cells was negligible under the employed conditions (data not shown). Though the production of sBAFF from normal T cells reached to 6.2 ± 2.9 ng ml⁻¹ upon stimulation, this elevation was not statistically significant. Remarkably, stimulated SLE T cells produced ~5-fold more abundant sBAFF (43.0 ± 4.9 ng ml⁻¹) than those under basal conditions (Fig. 2a). The strong induction of BAFF in SLE T cells was confirmed by RT-PCR (Fig. 2b, 'none' and '+CD3'). Mobility of the amplified band through the gel was same as that of a fragment which was amplified using the control plasmid, pTarget/FL-BAFF, as a template (Fig. 2b, 'C'). Therefore, it is likely that enhanced secretion of sBAFF from SLE T cells was a consequence of the induction of gene expression. In addition, these results clearly indicate that BAFF is also expressed in peripheral T cells.

Inducible expression of BAFF in Loucy
In order to investigate the expression of BAFF in T cells in more detail, we searched for human T cell lines which show inducible expression of BAFF. For this purpose, we used TPA + ionomycin (T1), which mimics stimulation to T cells via TCR, as a stimulus, because our preliminary experiments had shown that T1 had a stronger effect than the anti-CD3 antibody (data not shown). The cells were cultured for 48 h in the absence or presence of T1, and the expression of BAFF was examined by RT-PCR. Among T cell lines thus far examined, a T cell line named Loucy, which was previously established from T cell acute lymphoblastic leukemia (30), showed robust induction of BAFF in response to the stimulation. On the other hand, other T cell lines as well as a human mononuclear cell line, THP-1, expressed BAFF almost constitutively (Fig. 3a). Like peripheral T cells, Loucy also produced IFN-γ, IL-2 and TNF-α upon stimulation with T1 (Fig. 3b).

Inducible expression of BAFF in Loucy was further investigated by real-time PCR, western blot analysis and sandwich ELISA. Doubling time of Loucy was ~40 h under our culture conditions, and its growth rate was not significantly affected by the stimulation with T1 (data not shown). Real-time PCR
analysis revealed that expression of the BAFF gene was induced by ~15-fold upon stimulation and reached a plateau after 24 h (Fig. 4a). Western blot analysis of cell lysate with an anti-BAFF antibody detected a single band of ~42 kDa, which corresponds to human BAFF (Fig. 4b, 0 h). The production of BAFF in Loucy was markedly enhanced when the cells were stimulated with TI (Fig. 4b, 24 and 48 h). In accordance with these data, the cumulative amount of sBAFF in the medium was largely increased when the cells were cultured for 48 h in the presence of TI (Fig. 5a).

**Regulation of the expression of BAFF**

Like peripheral SLE T cells, Loucy produced considerable amount of sBAFF in response to the stimulation through TCR (Fig. 5b), suggesting that Loucy provides a model system to explore the mechanism of inducible expression of BAFF in SLE T cells. It is well known that stimulation via TCR triggers signal transduction through the mitogen-activated protein kinase (MAPK) (JNK/p38) cascade (31). We investigated if this pathway is also responsible for the induction of BAFF in Loucy. SP600125, a selective inhibitor of JNK, inhibited the production of sBAFF in a dose-dependent manner (Fig. 6a). Similarly, SB203580, which specifically inhibits p38, also suppressed the production of sBAFF (Fig. 6b). Neither of the compounds significantly affected the growth of the cells (Fig. 6a and b). Taken together, it is likely that at least the MAPK pathway is involved in the inducible expression of BAFF in Loucy.

It has been reported that IFN-γ is implicated in the induction of BAFF in monocytes and dendritic cells (32–35). In accordance with these previous observations, expression of sBAFF in Loucy was also significantly enhanced by IFN-γ in a dose-dependent manner (Fig. 7a). RT-PCR analysis revealed that expression of the BAFF gene was induced by ~15-fold upon stimulation and reached a plateau after 24 h (Fig. 4a). Western blot analysis of cell lysate with an anti-BAFF antibody detected a single band of ~42 kDa, which corresponds to human BAFF (Fig. 4b, 0 h). The production of BAFF in Loucy was markedly enhanced when the cells were stimulated with TI (Fig. 4b, 24 and 48 h). In accordance with these data, the cumulative amount of sBAFF in the medium was largely increased when the cells were cultured for 48 h in the presence of TI (Fig. 5a).

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Possible involvement of furin in the release of sBAFF

Schneider et al. (3) suggested that furin, a membrane-bound protease of subtilisin family (36–38), was responsible for the cleavage of BAFF to release sBAFF. In order to examine if this is also the case with T cells, we applied a specific furin inhibitor, decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone, to the culture of Loucy. The release of sBAFF from the cells was considerably suppressed by the inhibitor, while the compound did not affect the growth of the cells (Fig. 8). In addition, expression of the BAFF gene was not affected by the compound either (data not shown). These data strongly suggest that furin is one of the proteases which are involved in the shedding of BAFF.

Discussion

In this study, we prepared a mAb which specifically recognizes human sBAFF and developed an ELISA system to measure its concentration (Fig. 1). We revealed that peripheral T cells produced detectable amount of sBAFF in vitro under unstimulated conditions (Fig. 2a). According to our data, SLE T cells produced more sBAFF than normal T cells. However, since patients and normal subjects were not completely age matched, a possibility that difference in age affected the production of sBAFF may not be ruled out. When normal T cells were stimulated with an anti-CD3 antibody, a slight increase in the production of sBAFF was observed. However, the elevation was only marginal and statistically insignificant. In marked contrast to normal T cells, SLE T cells showed drastic increase in the production of sBAFF upon stimulation. The degree of response to the stimulus varied among patients, who had distinct clinical manifestations, disease activities and treatment regimens (Fig. 2a). RT-PCR analysis demonstrated that this increase was a consequence of induction of gene expression (Fig. 2b). These data clearly indicate that not only monocytes and dendritic cells but also T cells can produce BAFF thorough signal transduction pathways triggered by
antigens. In addition, these data imply that aberrant production of sBAFF from SLE T cells upon stimulation through TCR may have some relevance to the development of the disease.

The fact that the response of SLE T cells to the stimulus was variable among patients made it difficult to analyze the molecular mechanism of BAFF production in T cells. One solution to solve this problem was to obtain a cell line which consistently showed inducible expression of BAFF in response to stimuli. In this context, we screened several human T cell lines and found that a cell line established from T cell acute lymphoblastic leukemia named Loucy met the criteria (Fig. 3a and b). Several lines of evidence indicated that BAFF was robustly induced in Loucy when the cells were stimulated with T1, which mimics stimulation to T cells via TCR (Fig. 4a and b).

Fig. 6. Involvement of MAPK pathways in the expression of BAFF in Loucy. Loucy (2.5 × 10^5 ml⁻¹) was cultured for 48 h in the presence of TPA (50 ng ml⁻¹) + ionomycin (1 μg ml⁻¹). The cells were simultaneously exposed to 0–10 μM of SP600125 (a) or SB203580 (b), and the amounts of sBAFF in the culture supernatants were measured by ELISA (n = 4). Viabilities of the cells were estimated by MTT assay (n = 10). Data represent mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

Notably, the production of sBAFF was also significantly induced in Loucy when the cells were treated with an anti-CD3 antibody (Fig. 5b). Relatively high background (Fig. 5b) as compared with the T1 stimulation (Fig. 5a) might be caused by non-specific stimulation by the control IgG under the employed conditions. It is likely that MAPK pathway is one of the signal transduction cascades via TCR which is responsible for the production of sBAFF in Loucy (Fig. 6a and b). It is known that TCR triggers release of calcium from intracellular calcium store, which results in activation of a transcription factor, nuclear factor of activated T cells (NFAT) (39). Since TPA was unable to induce the expression of BAFF without ionomycin (data not shown), it appears that NFAT is also involved in the expression of BAFF.

It may be noteworthy that another pathway primed by IFN-γ (32–35) is involved in the regulation of the expression of BAFF in Loucy (Fig. 7a and b). Accordingly, it seems that a Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway is also implicated in the regulatory mechanism. These data are consistent with the reports that the serum level of IFN-γ was elevated in SLE patients (40–45). As...
IFN-γ directly regulates the differentiation of B cells, these data suggest that abnormal elevation of serum IFN-γ in SLE patients may exert its functions to B cells through at least two distinct pathways, that is, direct activation of B cells and indirect stimulation of B cells by enhancing the production of BAFF from not only macrophages (1) but also T cells.

Regarding processing of BAFF, a membrane-bound pro- tease, furin, was involved in the shedding of sBAFF in Loucy (Fig. 8), which was consistent with the results reported by another group (3).

In conclusion, all the data reported in this study strongly suggest that Loucy recapitulates some of the characteristics of SLE T cells. Thus, Loucy may provide not only a cellular model to investigate the mechanism of abnormal production of BAFF in SLE T cells but also a useful tool to explore therapeutic possibilities to treat the diseases.

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Abbreviations

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<tr>
<td>ATCC</td>
<td>American Type Tissue Collection</td>
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<tr>
<td>BAFF</td>
<td>B cell-activating factor belonging to the tumor necrosis factor family</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>JNK</td>
<td>c-Jun NH2-terminal kinase</td>
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<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>NFAT</td>
<td>nuclear factor of activated T cells</td>
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<td>RT</td>
<td>reverse transcription</td>
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<td>sBAFF</td>
<td>soluble BAFF</td>
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<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
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<tr>
<td>TI</td>
<td>TPA + ionomycin</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>TPA</td>
<td>12-O-tetradecanoyl-phorbol-13-acetate</td>
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<td>TTBS</td>
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References

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