Functional implication of BAFF synthesis and release in gangliosides-stimulated microglia

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ABSTRACT

BAFF is a recently identified member of the TNF ligand superfamily that plays a critical role in B cell differentiation, survival, and regulation of Ig production. In the present study, we examined whether BAFF is expressed in microglia, and the expression and release of BAFF are regulated by gangliosides. The results showed that BAFF was expressed and released in rat primary microglia as well as in BV-2 cells. Furthermore, its expression and release were increased by gangliosides stimulation and regulated by JAK-STAT, especially the STAT1- and STAT3-dependent signaling pathways. It was of particular interest to observe that SP600125 and SB203580, specific inhibitors of JNK and p38, did not inhibit BAFF synthesis but inhibited the release of sBAFF in gangliosides-treated cells by regulating furin expression, suggesting that the JNK and p38 signaling pathways regulate the release but not the synthesis of BAFF. Moreover, BV-2 cells expressed BAFF-R on their cell surface, and rat primary microglia expressed BAFF-R and TACI on their cell surface. rBAFF increased the release of cytokines, especially IL-6, TNF-α, and IL-10, in rat primary microglia as well as in BV-2 cells. These findings imply that BAFF secreted by microglia may play important roles in CNS inflammation by regulating microglia as well as infiltrated cells.

Introduction

BAFF (also known as BlyS, TALL-1, THANK, zTNF4, or TNFSF13B) is a recently identified member of the TNF ligand superfamily that plays a critical role in B cell differentiation, survival, and regulation of Ig production [1–5]. It is found on the cell surface as a type II transmembrane protein, or it can be released in a soluble form after cleavage [1, 2, 4] and has been known to be produced by macrophages, monocytes, DCs, activated T cells, and neutrophils [6–8]. Three BAFF-Rs have been identified so far: BAFF-R, TACI, and BCMA [9–11]. These receptors are primarily expressed in B-lineage cells; however, a subset of T cells has also been shown to express TACI and BAFF-R [12, 13].

The role of BAFF in humoral immune response has been shown in BAFF-deficient mice, which have deficiencies in peripherical B cell development and maturation [14]. Transgenic mice overexpressing BAFF are shown to develop autoimmune disorders that are similar to SLE in humans [15, 16]. Consistent with these observations, BAFF levels are higher in human autoimmune diseases, such as SLE, Sjögren syndrome, and rheumatoid arthritis [17–20]. In addition to autoimmune diseases, malignant B cells from non-Hodgkin’s lymphoma and multiple myeloma patients also express abnormal levels of BAFF protein, which plays a role in survival of malignant cells [21, 22]. These findings suggest that specific targeting of BAFF could provide a novel treatment modality for these diseases.

Nevertheless, the mechanisms for regulating BAFF expression and release have not yet been fully understood. Cytokines such as IL-10, IFN-γ, and IFN-α are shown to augment BAFF expression in various cell types such as monocytes, macrophages, and DCs [23], and IL-4 has been reported to inhibit the up-regulation of BAFF expression in monocytes stimulated with IL-10 but not with IFN-γ [24]. Recently, constitutive NF-κB and NFAT activations have been shown to be crucial transcriptional regulators of the BAFF survival pathway in malignant B cells [25]. However, as a result of the known expression pattern of its receptors in B cells, the roles of BAFF in other cells have not been addressed adequately. Recently, Chang et al. [26] reported that monocytes express TACI and that rBAFF induces monocyte survival, activation, and differentiation into macrophage-like cells, suggesting that BAFF may regulate the function of other immune cells in addition to B or T immune cells.

Microglia are the resident macrophages of the CNS and immunological competent cells whose functions include chemotaxis, phagocytosis, antigen presentation, and secretion of a...
variety of cytokines and proteases [27]. They are activated in brain injuries and several neurodegenerative and autoimmune diseases in the CNS such as Alzheimer’s disease [28], Parkinson’s disease [29], and multiple sclerosis [30]. Therefore, they are considered to be a key player in brain inflammation [31]. Krumholz et al. [32] recently detected BAFF in brain tissue and cerebrospinal fluid and also demonstrated that it was in part produced locally, that BAFF-producing cells might be astrocytes, evidenced by the immunopathological observation, and that CNS-produced BAFF might support B cell survival in CNS inflammatory diseases and primary B cell lymphoma.

In the present study, we examined whether BAFF is also expressed in microglia, and the expression and release of BAFF are regulated by gangliosides, which are sialic acid-containing glycosphingolipids and are constituents of mammalian cell membranes, are particularly abundant in neuronal cell membranes, and participate in various cellular events of the nervous system, such as proliferation, adhesion, and differentiation [33–35]. In addition, gangliosides have been known to induce the production of various inflammatory mediators, such as cytokines and inducible NO synthase [36–38]. We also reported previously that brain Gmix stimulates microglia via a variety of signaling pathways, especially Jak-STAT and MAPK signaling pathways [37, 39], and triggers inflammatory responses via TLR4 [40]. We used Gmix in the present study as a stimulus to elucidate which signaling pathways are important in regulation of BAFF expression in microglia. Also, to examine whether BAFF influences the microglial activation, we examined three types of BAFF-Rs and cytokine release by rsBAFF in microglia.

**MATERIALS AND METHODS**

**Reagents**

Gmix was purchased from Matreya (Pleasant Gap, PA, USA). MAPK inhibitors (PD98059, SB203580, and SP600125) and JAK2 were purchased from Calbiochem (San Diego, CA, USA). Oligodeoxycytid and Lipopolysaccharide Plus reagent were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). The siRNA against mouse STAT-1 and STAT-3 were purchased from Dharmacon Research (Lafayette, CO, USA) in deprotected and de-oxycytid form. The ELISA kit for mouse BAFF was purchased from Apootech (Lausen, Switzerland): The lowest level of BAFF that can be detected by this assay is 0.2 ng/ml. Rabbit polyclonal antibody against furin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat polyclonal antibodies against mouse BAFF, mouse BAFF-R, mouse TACI, and mouse BCMA were purchased from R&D Systems (Minneapolis, MN, USA). Mouse rsBAFF was purchased from R&D Systems. ELISA kits for mouse IL-6, mouse TNF-α, mouse IL-10, rat IL-6, rat TNF-α, and rat IL-10 were purchased from BD PharMingen (San Diego, CA, USA): The lowest levels of these cytokines that can be detected are 4 pg/ml, 4 pg/ml, 4 pg/ml, 15 pg/ml, and 4 pg/ml, respectively.

**Cell culture**

BV-2 cells, immortalized murine microglial cell line, were grown in DMEM supplemented with 5% FBS. Primary microglia from the cerebral cortices of 1-day-old Sprague-Dawley rats were cultured as described previously [37, 39]. Briefly, the cortices were triturated into single cells in MEM containing 10% FBS and were plated into 75 cm² T-flasks for 2 weeks. Microglia were then detached from the flasks by mild shaking and filtered through a nylon mesh to remove astrocytes. Cells were then plated into six-well plates or 60-mm dishes for use in subsequent experiments. The purity of primary microglia was determined by flow cytometry using anti-ox-42 antibody (Se-rotec, Kidlington, UK), and its approximate percentage was 93–95% of total cultured cells (data not shown).

**RT-PCR analysis**

Total RNA was isolated using RNAsol B (Intron, Seoul, Korea), and cDNA was prepared using avian myeloblastosis virus RT (Promega, Madison, WI, USA) according to the manufacturer’s instructions. PCR was performed with 30–32 cycles of sequential reaction as follows: 94°C for 30 s, 56–58°C for 30 s, and 72°C for 90 s. Oligonucleotide primers were purchased from Bioneer (Daejeon, Korea). The oligonucleotide sequences were as follows: forward, 5’-CTCGTGTAACTCACAAGCTGCTG-3’, and reverse, 5’-GACGCTGTGGTTACTAGCTACAAA-3’, for mouse and rat BAFF; forward, 5’-TCCCCAAC- GATTGTAGCAGCAA-3’, and reverse, 5’-AGATCCACACCGGATACATT-3’, for rat GAPDH; forward, 5’-GCCATCTCTGACTGGAAGTC-3’, and reverse, 5’- CATGTTTGGAGACCTTCAAC-3’, for mouse actin.

**Confocal microscopy**

Rat primary microglia cultured on poly-D-lysine coated coverslips were incubated in several reagents, washed twice with PBS, and fixed in ice-cold 100% methanol. The fixed cells were then washed in PBS and incubated with PBS containing 0.1% Triton X-100 for 10 min at room temperature. After washing several times with PBS, the cells were blocked with PBS containing 5% BSA for 30 min at room temperature and then incubated overnight with the indicated antibodies at 4°C. Preparations were then washed with FITC- and rhodamine-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, USA) for 2 h, mounted, and observed under a confocal microscope (Zeiss, Germany).

**Transfection**

BV-2 cells were transfected with siRNA oligonucleotides using oligo- fectamine (Invitrogen Life Technologies), according to the manufacturer’s instructions. After 24 h of transfection, the cells were washed with PBS and then treated with 100 μg/ml Gmix for 6 and 36 h for RT-PCR and ELISA analysis, respectively, unless stated otherwise.

**Flow cytometry**

BV-2 cells were dispersed into a single-cell suspension with PBS. To detect BAFF, BAFF-R, TACI, and BCMA expressions on cell surface, cells were blocked by 3% BSA containing PBS for 1 h at 4°C and then incubated with primary antibodies for 1 h at 4°C. After washing three times with PBS, cells were incubated with FITC-conjugated secondary antibody (Jackson Immunoresearch Laboratories) for 1 h at 4°C. After washing, they were analyzed using a FACSvantage (Becton Dickinson, Franklin Lakes, NJ, USA). To detect intracellular BAFF, BAFF-R, TACI, and BCMA, cells were fixed with 4% paraformaldehyde and were permeablized using PBS containing 0.1% Triton X-100. The next procedures were the same as those used for the above cell surface staining. Data were analyzed with WinMDI software (The Scripps Research Institute, La Jolla, CA, USA).

**Western blot**

After treatment, BV-2 cells were lysed in ice-cold radioimmununoprecipitation assay buffer (50 mM Tris-HCl pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, and 1 mM Na_2VO_4) containing protease inhibitors (2 mM PMSF, 100 μg/ml leupeptin, 10 μg/ml pepstatin, 1 μg/ml aprotinin, and 2 mM EDTA). Mouse PBMC was obtained from mouse spleen. The lysates were centrifuged at 12,000 g for 10 min at 4°C, and the supernatant was collected. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were incubated with BAFF antibody or furin antibody and HRP-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA, USA). They were then visualized using an ECL system (Sigma Chemical Co., St. Louis, MO, USA).
ELISA
BAFF, IL-6, TNF-α, and IL-10 levels in cell-free culture supernatants were determined using cytokine-specific ELISA kits according to the manufacturer’s instructions. All samples were calibrated from standards containing known concentrations of the cytokines.

RESULTS
Gmix stimulates microglia to express BAFF in dose- and time-dependent manners
To evaluate whether microglia express BAFF and its expression is increased by Gmix, BV-2 cells were treated with Gmix. Semiquantitative RT-PCR and ELISA analyses were then performed. We used BV-2 cells initially, as they mimic behavior of primary microglia [41, 42], and we had to rule out BAFF expression as a result of a few primary astrocytes [32] contaminated in primary microglial culture. As shown in Figure 1, BAFF was expressed in resting BV-2 cells, and its expression was increased by Gmix, which increased the mRNA level of BAFF and the release of sBAFF in BV-2 cells in dose- and time-dependent manners. Similar to BV-2 cells, rat primary microglia in resting states also expressed BAFF, and Gmix increased the mRNA level of BAFF and the release of sBAFF (Fig. 2, A and B). We also confirmed these results by confocal microscopy. Iba-1 has been described previously to be specific for microglial cells [43, 44]. Figure 2C shows that Iba-1-positive microglia expressed BAFF in resting and Gmix-stimulated

Figure 1. Gmix-stimulated BV-2 cells express and release BAFF in dose- and time-dependent manners. BV-2 cells were incubated with 100 μg/ml Gmix for the indicated time. Then, RT-PCR (A) for the detection of the mRNA of BAFF and ELISA (C) for the detection of the release of sBAFF were performed. BV-2 cells were incubated with the indicated dose of Gmix. Then, RT-PCR (B) and ELISA (D) were performed at 6 h and 36 h after treatment, respectively. (C and D) Values are mean ± sem of three replicates. Data shown are representatives of three experiments. **, P < 0.01, against no treatment.

Figure 2. Gmix-stimulated rat primary microglia express and release BAFF. Rat primary microglia were incubated with 100 μg/ml Gmix for the indicated time (A, upper panel) or with the indicated dose of Gmix for 6 h (A, lower panel). Then, RT-PCR was performed. (B) Rat primary microglia were incubated with 100 μg/ml Gmix for 36 h. ELISA was then performed. Values are mean ± sem of three replicates. Data shown are representatives of three experiments. **, P < 0.01. (C) Rat primary microglia were incubated in the absence or presence of 100 μg/ml Gmix for 24 h. The cells were then stained with anti-BAFF (green) and anti-Iba-1 (red) antibodies, as described in Materials and Methods, and examined by confocal microscopy. (-), Staining pattern without primary antibodies. Blue indicates DAPI staining. Original scale bar, 10 μm.
conditions. However, the BAFF level was not different between resting and Gmix-treated rat primary microglia, suggesting that the increase of BAFF level by Gmix was a result of immediate release as a soluble form. These results suggest that microglia in a resting state express BAFF and that Gmix increases BAFF expression in microglia.

### BAFF expression in Gmix-treated BV-2 cells is mediated by the STAT1 and STAT3 signaling pathways

We reported previously that Gmix stimulates the JAK-STAT signaling pathway in microglia [39]. To elucidate whether the increase of the BAFF mRNA level and sBAFF release in Gmix-treated BV-2 cells was mediated by the JAK-STAT signaling pathway, we performed the following experiments. Initially, Gmix-treated BV-2 cells were preincubated with JAKi, a broad-spectrum JAKi [45]. As shown in Figure 3, A and D, JAKi inhibited the increase of BAFF mRNA and sBAFF release efficiently. The transfection of siRNA against STAT1 and STAT3 inhibited the expression of STAT1 and STAT3 efficiently (Fig. 3B) and also inhibited the increase of BAFF mRNA and sBAFF release in Gmix-treated BV-2 cells, respectively (Fig. 3, C and E). These results suggest that the increase of BAFF expression in microglia by Gmix is mediated by JAK-STAT, especially the STAT1- and STAT3-dependent signaling pathways.

### The release of sBAFF in Gmix-treated BV-2 cells is dependent on the p38 and JNK signaling pathways

We reported previously that Gmix activates MAPK signaling pathways in microglia [37]. To elucidate whether MAPK signaling pathways also mediate the expression and release of BAFF in microglia, we used several MAPK inhibitors: PD98059, SP600125, and SB203580. As shown in Figure 4, p38 and JNK do not regulate the synthesis of BAFF, however, inhibit the increase of sBAFF release in Gmix-treated BV-2 cells. BV-2 cells were incubated with 100 nM Jaki for 1 h and treated with 100 µg/ml Gmix for 6 h (RT-PCR) or 36 h (ELISA). Then, RT-PCR and ELISA were performed. (A) BV-2 cells were transfected with siRNAs (C and E) indicated. After 24 h, the cells were lysed. Cell lysates were resolved on SDS-PAGE gels and blotted with anti-STAT1 and anti-STAT3 antibodies, respectively. con, Control siRNA. (D) Values are mean ± sem of three replicates. Data shown are representatives of three experiments. *, P < 0.05; **, P < 0.01.
a selective inhibitor of ERK; SP600125, a selective inhibitor of JNK; and SB203580, a selective inhibitor of p38. After preincubation with MAPK inhibitors for 1 h, BV-2 cells were treated with Gmix. As shown in Figure 4A, the increased level of BAFF mRNA in Gmix-treated BV-2 cells was hardly changed by PD98059, SP600125, or SB203580. Surprisingly, however, the release of sBAFF from Gmix-treated BV-2 cells was inhibited by SP600125 and SB203580 (Fig. 4B), and these results were highly reproducible. Therefore, it is highly possible that the JNK and p38 signaling pathways are associated with extracellular release of sBAFF in Gmix-treated BV-2 cells.

To further confirm the above contention, we performed flow cytometric analysis to monitor cytoplasm and membrane-bound BAFF levels in Gmix-treated BV-2 cells, with and without preincubation with MAPK inhibitors. As shown in Figure 5, resting BV-2 cells expressed BAFF on the cell surface as a membrane-bound form, and the BAFF level was hardly different between resting and Gmix-treated BV-2 cells, suggesting that the increase of BAFF by Gmix was a result of its immediate release as a soluble form, similar to those observed in Gmix-treated rat primary microglia by confocal microscopy (Fig. 2C). On the other hand, BV-2 cells that were preincubated with SP600125 or SB203580 showed that their BAFF level increased in the cellular compartment (Fig. 5). Membrane-bound BAFF levels were also increased by inhibition of the JNK and p38 signaling pathways. We also confirmed these observations by Western blot analysis (Fig. 6A) and confocal microscopic analysis (Fig. 6B). It has been known that sBAFF release is mediated by furin [4, 32, 46, 47]. Therefore, to evaluate whether the JNK and p38 signaling pathways are involved in furin expression, we performed Western blot analysis. As shown in Figure 6C, furin expression was increased by Gmix, and SP600125 and SB203580 inhibited the furin expression induced by Gmix, suggesting that JNK and p38 seem to mediate the release of sBAFF in Gmix-treated BV-2 cells by regulating furin expression.

**BV-2 cells express BAFF-R but not TACI and BCMA, whereas rat primary microglia express BAFF-R and TACI but not BCMA**

To elucidate whether the expression and release of BAFF in microglia could affect their own status, we studied the expressions of three BAFF-Rs (BAFF-R, TACI, and BCMA) in BV-2 cells by flow cytometry. Interestingly, BV-2 cells expressed BAFF-R but not TACI or BCMA. As shown in Figure 7, A and B, BAFF-R was detected on the cell surface and in the cytoplasm. However, TACI and BCMA were not detected in the cytoplasm or the cell surface. We observed similar results by RT-PCR analysis (data not shown).

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**Figure 5. p38 and JNK regulate the release of sBAFF from Gmix-treated BV-2 cells, which were incubated with 2 μM PD98059, 2 μM SP600125, or 2 μM SB203580 for 1 h, respectively, and treated with 100 μg/ml Gmix for 24 h. After treatment, cells were harvested. Then, flow cytometry was performed. Representative results from flow cytometric analysis to detect cell surface BAFF in nonpermeabilized BV-2 cells (A) and cell-associated BAFF in permeabilized BV-2 cells (B), which were treated with inhibitors indicated. Dotted lines indicate irrelevant antibody binding, and solid lines indicate BAFF expression in resting BV-2 cells. Solid lines filled with gray indicate BAFF expression of Gmix-treated BV-2 cells in the presence or absence of indicated inhibitors. The graphs represent the mean fluorescence intensity of BAFF in nonpermeabilized and permeabilized BV-2 cells, respectively. Values are mean ± SEM of two replicates. *, P < 0.05; **, P < 0.01.**
Figure 6. p38 and JNK regulate the expression of furin in Gmix-treated BV-2 cells, which were incubated with 2 μM PD98059, 2 μM SP600125, or 2 μM SB203580 for 1 h, respectively, and then treated with 100 μg/ml Gmix for 24 h. After treatment, the cells were harvested. Then, Western blot analysis for BAFF (A) and furin (C) was performed. Mouse PBMC was used as a positive control for BAFF. The graph represents the fold induction of furin in Western blot analysis. (B) The cells were then stained with anti-BAFF (green) and anti-OX-42 (red), as described in Materials and Methods, and examined by confocal microscopy. Blue indicates DAPI staining. Original scale bar, 10 μm. *, P < 0.05; **, P < 0.01.

Figure 7. BV-2 cells express only BAFF-R, but rat primary microglia express BAFF-R and TACI. Cultured BV-2 cells (A and B) and rat primary microglia (C and D) were harvested. Then, flow cytometry was performed to detect indicated receptors, BAFF-R, TACI, and BCMA. Solid lines filled with gray indicate primary antibody binding, and solid lines without filling indicate irrelevant antibody binding. Data are representatives of flow cytometric analysis in nonpermeabilized (A and C) and permeabilized (B and D) microglia and also representatives of three experiments. (E) Rat primary microglia were stained with anti-BAFF-R, -TACI, or -BCMA antibodies (green) and -Iba-1 (red) antibody, as described in Materials and Methods and examined by confocal microscopy. Blue indicates DAPI staining. Original scale bar, 10 μm.
We also performed flow cytometry to evaluate whether rat primary microglia also express BAFF-Rs. Rat primary microglia expressed BAFF-R on the cell surface and cytoplasm (Fig. 7, C and D). Interestingly, however, unlike BV-2 cells, rat primary microglia expressed TACI as well as BAFF-R on the cell surface and cytoplasm. BCMA was not expressed in rat primary microglia as in BV-2 cells (Fig. 7, C and D). We also obtained similar results by RT-PCR analysis (data not shown) and confocal microscopic analysis (Fig. 7E), suggesting that microglia express the receptors for BAFF and that BAFF might regulate the function of microglia.

BAFF promotes cytokine release in BV-2 cells and in rat primary microglia

As shown above, we observed that microglia expressed the receptors for BAFF, although BV-2 cells and rat primary microglia expressed a different set of BAFF-Rs. Therefore, it is highly likely that sBAFF might regulate the function of microglia. To elucidate the possibility, we assayed the release of pro- and anti-inflammatory cytokines in rat primary microglia as well as in BV-2 cells by rsBAFF. As shown in Figure 8, sBAFF increased IL-6, TNF-α, and IL-10 secretion in BV-2 cells and rat primary microglia in a dose-dependent manner. To further confirm these observations, we performed these experiments in the presence of neutralizing antibodies. In BV-2 cells, sBAFF-induced cytokine secretion was inhibited by anti-BAFF-R antibody but not by anti-TACI antibody. In rat primary microglia, however, they were inhibited by anti-BAFF-R and -TACI antibodies, suggesting that sBAFF-induced cytokine secretion is dependent on the status of its receptors’ expression (Fig. 9).

Consistent with our previous report [37], Gmix induced TNF-α and IL-6 secretion in microglia. Gmix-induced cytokine secretion was also inhibited by anti-BAFF-R or -TACI antibodies in their receptor expression-dependent manner (Fig. 9), suggesting that sBAFF secreted in the CNS might regulate the function of microglia in the condition of inflammatory disease as well as resting state.

**DISCUSSION**

Recently, BAFF has been known to be expressed by human astrocytes [32] and airway epithelial cells [48] in addition to cells previously known to express it, such as macrophages, monocytes, DCs, activated T cells, and neutrophils. In the present study, we demonstrated that microglia also express and secrete BAFF. We also showed that Gmix stimulated BAFF expression and release in microglia: Gmix efficiently increased the mRNA level of BAFF and the release of sBAFF in microglia in time- and dose-dependent manners. It should be mentioned that we used a mouse BAFF ELISA kit to detect rat sBAFF, which was secreted in rat primary microglia, because of a high degree of interspecies homology between mouse and rat BAFF.

Figure 8. Release of IL-6, TNF-α, and IL-10 is increased in BV2 cells and rat primary microglia by rsBAFF. BV-2 cells and rat primary microglia were stimulated with indicated doses of rsBAFF for 24 h. IL-6, TNF-α, and IL-10 in cell-free supernatants of BV2 cells (A–C) and rat primary microglia (D–F) were then analyzed by specific ELISA kits. N.D. Not detected. Values are mean ± sem of two or three replicates. *, P < 0.05; **, P < 0.01, against no treatment.

Gangliosides are constituents of mammalian cell membranes and are particularly abundant in neuronal cell membranes. Thus, gangliosides could be released from damaged cells to the extracellular space in injured brain. Several studies indicate that the amount of gangliosides in cerebrospinal fluid increases in neurodegenerative diseases such as Alzheimer’s disease [49] and multiple sclerosis [50], and Krumbholz et al. [32] demonstrated that BAFF is up-regulated in multiple sclerosis lesions. Together with our present finding, it is quite possible that microglia are activated by increased gangliosides, consequently secreting more BAFF in these inflammatory diseases.

Following this determination, we attempted to uncover the molecular mechanism of the regulation of BAFF expression in Gmix-stimulated microglia and found that the expression and release of BAFF in microglia were regulated by the JAK-STAT signaling pathway. The inhibition of STAT1 and STAT3 by transfection with siRNA against STAT1 and STAT3 also attenuated the increase of BAFF expression and secretion in Gmix-treated microglia. These findings are partly
consistent with the results on human epithelial cells by Kato et al. [48]. In the present study, we could not investigate the role of other STAT proteins in the expression and secretion of BAFF in microglia, as it was not certain whether Gmix activates other STAT proteins in microglia. Further studies are required to determine whether other STAT proteins also play roles in the regulation of BAFF expression.

We observed the difference between semiquantitative RT-PCR and ELISA analyses in data about the effect of MAPK inhibitors on the expression and secretion of BAFF in Gmix-stimulated microglia. Yoshimoto et al. [47] reported that the inhibitors of p38 and JNK inhibited sBAFF production in 12-O-tetradecanoylphorbol 13-acetate- and ionomycin-stimulated Loucy, a human T cell line. However, they showed only sBAFF secretion by ELISA [47]. Therefore, to verify the difference in data between the two analytical methods, we additionally analyzed it by flow cytometry. Interestingly, we found that membrane-bound BAFF as well as cytosolic BAFF were increased in the presence of JNK and p38 inhibitors, suggesting that the JNK and p38 signaling pathways may regulate the secretion of sBAFF but not the de novo synthesis of BAFF. Furthermore, we also found that furin expression was regulated by the JNK and p38 signaling pathways in microglia, which is in agreement with another study [51]. The membrane-bound form of BAFF has been known to be processed by furin-like proteases [4, 32, 47], and furin expression has also been known to be related to sBAFF release [46]. Therefore, JNK and p38 might mediate intracellular processing of membrane-bound BAFF to soluble, secretable BAFF in microglia by regulating furin-like protease expression. However, we could not exclude another possibility that p38 and JNK might regulate intracellular trafficking of BAFF. Further study is needed to elucidate it.

To our best knowledge, we are the first to demonstrate that microglia express the receptors for BAFF. We inferred that locally produced BAFF in the CNS may regulate the function of other cells as well as infiltrated B cells, as reported recently by Chang et al. [26] and Krumbholz et al. [32]. Therefore, to elucidate this possibility, we examined the expression pattern of BAFF-Rs in BV-2 cells and rat primary microglia. Figure 7 shows that microglia expressed BAFF-Rs on the cell surface as well as in the cytoplasm. It is of interest to note that the expression patterns of BAFF-Rs were different between BV-2 cells and rat primary microglia: BV-2 cells expressed only BAFF-R, and rat primary microglia expressed BAFF-R and TACI. At present, we could not offer any explanation for the reason why the expression patterns of BAFF-Rs in BV-2 cells and rat primary microglia are different. It might be a result of the difference of species or the difference between cell line and primary cells. It might also be a result of the culture condition. In the case of rat primary microglia, we cultured the primary microglia along with other cells such as astrocytes and oligodendrocytes for 2 weeks before use, as described in Materials and Methods.

The fact that BAFF-Rs are expressed in microglia implies that BAFF may regulate the function of microglia. rsBAFF induced the secretion of IL-6 and TNF-α, representative proinflammatory cytokines, and IL-10, a representative anti-inflammatory cytokine, in BV-2 cells and rat primary microglia, re-
spectively, in a dose-dependent manner, suggesting that sBAFF may modulate the function of microglia. The observation that BAFF induced IL-6 and TNF-α secretion is consistent with the report of Chang et al. [26], who showed that BAFF induced IL-6, TNF-α, and IL-1β secretion in monocytes, evidenced by the fact that monocytes express TACI among BAFF-Rs. BAFF/BAFF-R interaction has been reported to be primarily responsible for B cell survival and responses [52–54]. However, the roles of BAFF/TACI interactions are open to controversy [55–58]. In the present study, the secretion of IL-6, TNF-α, and IL-10 in response to sBAFF was not different, although BV-2 cells and rat primary microglia expressed different sets of BAFF-Rs. Also, neutralizing antibodies against BAFF-R and TACI inhibited sBAFF-induced cytokine secretion significantly in rat primary microglia. Further studies are needed to elucidate the difference between BAFF-R and TACI signalings and other effects of BAFF on microglia in vivo. Furthermore, additional studies designed to reveal the association between BAFF and the function of microglia are expected to help develop therapeutic agents against several neurological diseases associated with inflammation.

In summary, our study demonstrated that BAFF is expressed and released in rat primary microglia as well as in BV-2 cells; the expression and release of BAFF in microglia are increased by Gmix stimulation; the expression and release of BAFF are regulated by JAK-STAT, especially by the STAT1- and STAT3-dependent signaling pathways; p38 and JNK signaling pathways regulate the release but not the synthesis of BAFF in microglia; BV-2 cells express BAFF-R, whereas rat primary microglia express BAFF-R and TACI; and sBAFF potentiates cytokines release, especially IL-6, TNF-α, and IL-10, in microglia. These results suggest that BAFF, secreted by microglia or astrocytes [32], may play important roles in CNS inflammation by regulating microglia as well as infiltrated B cells.

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REFERENCES


**KEY WORDS:**

BAFF-R, TACI