BAFF, a Novel Ligand of the Tumor Necrosis Factor Family, Stimulates B Cell Growth

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Summary
Members of the tumor necrosis factor (TNF) family induce pleiotropic biological responses, including cell growth, differentiation, and even death. Here we describe a novel member of the TNF family, designated BAFF (for B cell activating factor belonging to the TNF family), which is expressed by T cells and dendritic cells. Human BAFF was mapped to chromosome 13q32-34. Membrane-bound BAFF was processed and secreted through the action of a protease whose specificity matches that of the furin family of proprotein convertases. The expression of BAFF receptor appeared to be restricted to B cells. Both membrane-bound and soluble BAFF induced proliferation of anti-immunoglobulin M–stimulated peripheral blood B lymphocytes. Moreover, increased amounts of immunoglobulins were found in supernatants of germinal center–like B cells costimulated with BAFF. These results suggest that BAFF plays an important role as costimulator of B cell proliferation and function.

Key words: tumor necrosis factor • B lymphocytes • T lymphocytes • B cell growth • immunoglobulin G

Members of the TNF cytokine family are critically involved in the regulation of inflammation, of the immune response to infections, and of tissue homeostasis (1). The family members are type II membrane proteins that can act in a membrane-bound form or as proteolytically processed, soluble cytokines in an autocrine, paracrine, or endocrine manner (1). Binding of the ligands to their respective receptors induces oligomerization, initiating downstream signaling events.

Signaling pathways stimulated by TNF ligand members are diverse, including the activation of caspases, the translocation of nuclear factor (NF)–κB, or the activation of mitogen-activated protein kinases such as c-Jun N-terminal kinase (JNK) or extracellular signal regulatory kinase (ERK) (1). Thus, TNF-related ligands can lead to apoptosis, differentiation, or proliferation. Presently 16 members of the TNF cytokine family have been described, several among them having important regulatory roles in function and development of the immune system. For instance, TNF acts as an inflammatory cytokine coordinating host defenses in response to infection (2). The lymphotaxin (LT) system is crucial in the development of peripheral lymphoid organs and the organization of splenic architecture (3, 4). Fas ligand (FasL, CD95L), TNF, and CD30L are responsible for TCR-mediated apoptosis of T cells and of immature thymocytes (5–7). Several of the TNF members and their receptors in conjunction with TCR stimulation, also enhance T cell proliferation. Therefore, upregulation of TNF cytokine members and their receptors by TCR-induced signals can provide an autocrine costimulatory mechanism to increase the lymphocyte's own proliferation after stimulation with the antigen. However, upregulation of TNF-related ligands on T cells is also important for the activation

1Abbreviations used in this paper: aa, amino acid(s); APRIL, a proliferation-inducing ligand; BAFF, B cell activating factor belonging to the TNF family; EST, expressed sequence tag; LT, lymphotaxin; NF, nuclear factor; PNGase F, peptide N-glycanase F; RANKL, receptor activator of NF-κB ligand; TRAIL, TNF-related apoptosis-inducing ligand; wt, wild-type.
and stimulation of neighboring cells. For example, CD40L is important for B cell survival, proliferation, Ig isotype switch, and differentiation (8), and the interaction of OX 40 with OX 40L is necessary for the differentiation of activated B cells into high Ig-producing cells (9).

Here we characterize the structural and functional properties of a new ligand of the TNF cytokine family. The new ligand, termed BAFF (B cell activating factor belonging to the TNF family), appears to be expressed by T cells and dendritic cells for the purpose of B cell costimulation, and may therefore play an important role in the control of B cell function.

Materials and Methods

Materials. The anti-Flag M 2 mAb, biotinylated anti-Flag M 2 antibody, and the anti-Flag M 2 antibody coupled to agarose were purchased from Sigma Chemical Co. Cell culture reagents were antibody, and the anti-Flag M2 antibody coupled to agarose were purchased from PharMingen. Goat F(ab)2 labeled anti-CD4, anti-CD8, and anti-CD19 antibodies were either PharMingen or Jackson ImmunoResearch Laboratories. Secondary antibodies were obtained from anionic nucleotides AP1 and JT 1013 ('5'-ACT GTT TTCT CGACCC TGA ACC GC-3') using the provided cDNA library from a pool of human leukocytes as template, as recommended by the manufacturer. The resulting PCR product was cloned into PCR-0 blunt (Invitrogen) and subcloned as EcoR-I-PstI fragment into pT 7 T 3 Pac vector (Amersham Pharmacia Biotech) containing EST clone T 7 8 2 9. Therefore, full-length hBAFF cDNA was obtained by combining 5' and 3' fragments using the internal PstI site of hBAFF. The sequence has been assigned EM BLI/GenBank/DDBJ accession no. AF116456.

A partial 617-bp sequence of murine BAFF was contained in two overlapping EST clones (EMBL/GenBank/DDBJ) accession nos. AA422749 and AA254047. A PCR fragment spanning nucleotides 158-391 of this sequence was used as a probe to screen a mouse spleen cDNA library (Stratagene, Inc.). The sequence has been assigned EM BLI/GenBank/DDBJ accession no. AF119383.

Expression of Recombinant BAFF. Full-length hBAFF was amplified using oligos JT 1069 ('5'-GACAAGCTTGCCACCATG-GATGACTCCACA-3') and JT 637 ('5'-ACTGTACGAGC- GTTTCAATGC-3'). The PCR product was cloned into PCR-0 blunt and subcloned as HindIII-PstI fragment into PCR-3 mammalian expression vector. A short version of soluble BAFF (sBAFF/short, aa Q136-L285) was amplified using oligos JT 636 ('5'-CTGCAGGTCTCCAGAGAAACG-3') and JT 637. A long version of sBAFF (sBAFF/long, aa L83-L285) was obtained from full-length hBAFF using internal PstI site. sBAFFs were resubcloned as PstI-EcoRI fragments behind the hemagglutinating signal peptide and Flag sequence of a modified PCR-3 vector, and as PstI-SpeI fragments into a modified pQE16 bacterial expression vector in frame with an N-terminal Flag sequence (14). Constructs were sequenced on both strands.

The establishment of stable 293 cell lines expressing the short soluble form or full-length BAFF, and the expression and purification of recombinant sBAFF from bacteria and mammalian 293 cells were performed as described (14, 15).

Recombinant T cell receptor. Total RNA extracted from T cells, B cells, in vitro–derived immature dendritic cells, 293 wild-type (wt) and 293-BAFF (full-length) cells was reverse transcribed using the Ready to Go system (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. BAFF and β-actin cDNAs were detected by PCR amplification with Taq DNA polymerase (steps of 1 min each at 94°C, 55°C, and 72°C for 30 cycles) using specific oligonucleotides for BAFF, JT 1322 ('5'-GGAGAAGG- GCAACTCCAGTCAGAC-3') and JT 1323 ('5'-CAATTCAC-
specific IgGs. One of these mAbs, 43.9, is an IgG2a that specifically recognizes hBAFF.

FACS
d. Cells were stained in 50 μl of FACS buffer (PBS, 10% FCS, 0.02% NaN₃) with 50 ng (or the indicated amount) of Flag-tagged short soluble hBAFF for 20 min at 4°C, followed by anti-Flag M2 (1 μg) and secondary antibody. Anti-BAFF mAb 43.9 was used at 40 μg/ml. For two-color FACS® analysis, peripheral blood lymphocytes were stained with Flag-tagged sBAFF/long (2 μg/ml), followed by biotinylated anti-Flag M2 (1:400) and PE-labeled streptavidin (1:100), followed by FITC-labeled anti-CD4, anti-CD8, or anti-CD19.

PB L Proliferation Assay. PBLS were incubated in 96-well plates (10² cells/well in 100 μl RPMI supplemented with 10% FCS) for 72 h in the presence or absence of 2 μg/ml of goat anti-human μ chain antibody (Sigma Chemical Co.) or control F(ab)² and with the indicated concentration of native or boiled sBAFF/long. Cells were pulsed for an additional 6 h with [³H]thymidine (1 μCi/well) and harvested. [³H]Thymidine incorporation was monitored by liquid scintillation counting. In some experiments, recombinant sBAFF was replaced by 293 cells stably transfected with full-length BAFF (or 293 wild-type [wt] as control) that had been fixed for 5 min at 25°C in 1% paraformaldehyde. Assay was performed as described (17). In further experiments, CD19⁺ cells were isolated from PBLS with magnetic beads, and the remaining CD19⁻ cells were irradiated (3,000 rads) before reconstitution with CD19⁺ cells. Proliferation assay with sBAFF was then performed as described above.

B Cell Activation Assay. Purified B cells were activated in the EL-4 culture system as described (13). In brief, 10⁴ B cells mixed with 5 × 10⁴ irradiated murine EL-4 thymoma cells (clone B5) were cultured for 5–6 d in 200 μl medium containing 5% vol/vol of culture supernatants from human T cells (10⁶/ml) which had been activated for 48 h with PHA (1 μg/ml) and PWM (1 ng/ml). B cells were then reisolated with anti-CD19 beads and cultured for another 7 d (5 × 10⁴ cells in 200 μl, duplicate or triplicate

Figure 1. (A) Predicted aa sequence of human and mouse BAFF. The predicted transmembrane domain (TMD, dashed line), the potential N-linked glycosylation sites (stars), and the natural processing site of hBAFF (arrow) are indicated. The double line above hBAFF indicates the sequence obtained by Edman degradation of the processed form of hBAFF. (B) Comparison of the extracellular protein sequence of BAFF and some members of the TNF ligand family. Identical and homologous residues are represented in black and shaded boxes, respectively. (C) Dendrogram of TNF family ligands.

Purified B cells were stained in 50 μl of FACS buffer (PBS, 10% FCS, 0.02% NaN₃) with 50 ng (or the indicated amount) of Flag-tagged short soluble hBAFF for 20 min at 4°C, followed by anti-Flag M2 (1 μg) and secondary antibody. Anti-BAFF mAb 43.9 was used at 40 μg/ml. For two-color FACS® analysis, peripheral blood lymphocytes were stained with Flag-tagged sBAFF/long (2 μg/ml), followed by biotinylated anti-Flag M2 (1:400) and PE-labeled streptavidin (1:100), followed by FITC-labeled anti-CD4, anti-CD8, or anti-CD19.

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culture in flat-bottomed 96 well plates in medium alone or in medium supplemented with 5% T cell supernatants, or with 50 ng/ml IL-2 (a gift from the former Glaxo Institute for Molecular Biology, Geneva) and 10 ng/ml each IL-4 and IL-10 (Pepro-Tech), in the presence or absence of sBAFF. The anti-Flag M2 antibody was added at a concentration of 2 μg/ml and had no effect by itself.

Results

BAFF Is a Novel Ligand of the TNF Family. hBAFF was identified by sequence homology as a possible novel member of the TNF ligand family while we screened public databases using an improved profile search (18). A cDNA encoding the complete protein of 285 aa was obtained by combining EST clones (covering the 3' region) with a fragment (5' region) amplified by PCR. The absence of a signal peptide suggested that BAFF was a type II membrane protein that is typical of the members of the TNF ligand family. The protein has a predicted cytoplasmic domain of 46 aa, a hydrophobic transmembrane region, and an extracellular domain of 218 aa containing two potential N-glycosylation sites (Fig. 1 A). The sequence of the extracellular domain of BAFF shows highest homology with APRIL (33% aa identity, 48% homology), whereas the identity with other members of the family, such as TNF, FasL, LTα, TRAIL (TNF-related apoptosis-inducing ligand), or RANKL (receptor activator of NF-κB ligand) is <20% (Fig. 1, B and C). The mouse BAFF (mBAFF) cDNA clone isolated from a spleen library encoded a slightly longer protein (309 aa) due to an insertion between the transmembrane region and the first of several β strands which constitute the receptor binding domain in all TNF ligand members (19). This β strand-rich ectodomain is almost identical in mBAFF and hBAFF (86% identity, 93% homology), suggesting that the BAFF gene has been highly conserved during evolution (Fig. 1 A).

BAFF Is Processed and Secreted. Although TNF family members are synthesized as membrane-inserted ligands, cleavage in the stalk region between transmembrane and receptor binding domains is frequently observed. For example, TNF and FasL are readily cleaved from the cell surface by metalloproteinases (20, 21). While producing several forms of recombinant BAFF in 293 T cells, we noticed that a recombinant soluble 32-kD form of BAFF (aa 83–285, sBAFF/long), containing the complete stalk region and an N-linked glycosylation consensus site, was expressed fused to an NH2-terminal Flag tag and a 6-amino acid linker. The long form is cleaved between Arg 133 and Ala 134 (arrow) in 293 T cells, to yield a processed form of BAFF, Asn124 and Asn242, belonging to N-linked glycosylation consensus sites. N-linked glycan present on Asn242 is shown as a Y. (A) Schematic representation of recombinant BAFF constructs. Soluble recombinant BAFFs starting at Leu124 and Gln242 are expressed fused to an NH2-terminal Flag tag and a 6-amino acid linker. The long form is cleaved between Arg133 and Ala134 (arrow) in 293 T cells, to yield a processed form of BAFF. Asn124 and Asn242 belong to N-glycosylation consensus sites, N-linked glycan present on Asn242 is shown as a Y. TMD, transmembrane domain. (B) PNGase F treatment of recombinant BAFF. Concentrated supernatants containing Flag-tagged BAFFs and APRIL were deglycosylated and analyzed by Western blotting using polyclonal anti-BAFF antibodies or anti-Flag M2, as indicated. All bands except processed BAFF also reacted with anti-Flag M2 (data not shown). (C) Full-length BAFF is processed to a soluble form. 293 T cells were transfected with full-length BAFF. Transfected cells and their concentrated supernatants (S/N) were analyzed by Western blotting using polyclonal anti-BAFF antibodies. Supernatants corresponding to 10 times the amount of cells were loaded onto the gel. (D) Size exclusion chromatography of sBAFF on Superdex-200. Concentrated supernatants containing sBAFF/short were fractionated on a Superdex-200 column, and the eluted fractions were analyzed by Western blotting using anti-Flag M2 antibody. The migration positions of the molecular mass markers (in kD) are indicated on the left-hand side for SDS-PAGE and at the top of the figure for size exclusion chromatography.
corresponding to nondissociated dimers and trimers) were readily detectable in cellular extracts, but >95% of BAFF recovered from the supernatant corresponded to the processed 18-kD form, indicating that BAFF was also processed when synthesized as a membrane-bound ligand. Therefore, we engineered an sBAFF (Q136–L285, sBAFF/short) whose sequence started 2 aa downstream of the processing site (Fig. 1 B). As predicted, the Flag tag attached to the NH2 terminus of this recombinant molecule was not removed (data not shown), which allowed its purification by an anti-Flag affinity column. To test its correct folding, the purified sBAFF/short was analyzed by gel filtration where the protein eluted at an apparent molecular mass of 55 kD (Fig. 2 D). We conclude that sBAFF/short correctly assembles into a homotrimer (3 × 20 kD) in agreement with the quaternary structure of other TNF family members (19). Finally, unprocessed sBAFF/long was readily expressed in bacteria, indicating that the cleavage event was specific to eukaryotic cells.

Expression and Chromosomal Localization of BAFF. Northern blot analysis of BAFF revealed that the 2.5-kb BAFF mRNA was abundant in the spleen and PBLs (Fig. 3 A). Thymus, heart, placenta, small intestine, and lung showed weak expression. This restricted distribution suggested that cells present in lymphoid tissues were the main source of BAFF. Through PCR analysis, we found that BAFF mRNA was present in T cells and peripheral blood mono-
sis of umbilical cord and peripheral blood lymphocytes. While CD8+ and CD4+ T cells lacked BAFF-R (Fig. 4 B, and data not shown), abundant staining was observed on CD19+ B cells (Fig. 4, A and B), indicating that BAFF-R is expressed on all blood B cells, including naive and memory B cells. No evidence was obtained for a CD19+, BAFF-R2 population.

**Table I.** Binding of sBAFF to Various Cell Lines

| Cell type              | Cell lines | BAFF binding | Specific details         |
|------------------------|------------|--------------|--------------------------|
| Epithelial-like        | HT-29      | –            | Colon adenocarcinoma     |
| A375                   | –/+        | M melanoma   |
| MCF-7                  | –          | Breast adenocarcinoma |
| M e260                 | –          | M melanoma   |
| Cos                    | +          | Monkey kidney cells |
| Fibroblasts            | WI-38      | –            | Lung                     |
| Hs-68                  | –          | Foreskin     |
| Hs-27                  | –          | Foreskin     |
| Endothelial cells      | HUVEC      | –            | Umbilical vein           |
| M acrophages/monocytes | THP-1      | –/+          | Monocyte                 |
| T cell lines           | Molt-4     | –            | Lymphoblastic leukemia   |
| Hut-78                 | –          | Cutaneous lymphoma |
| Jurkat                 | –          | Lymphoblastic leukemia |
| B cell lines           | BJAB       | +++          | Burkitt lymphoma         |
| Namalawa               | ++         | Burkitt lymphoma |
| Daudi                  | +/-        | Burkitt lymphomaEBNA+VCA+ |
| R amsos                | ++         | Burkitt lymphomaEBV- |
| Raji                   | +++        | Burkitt lymphoma |
| JIYOYE                 | +          | Burkitt lymphoma |
| SKW.64                 | +++        | IgM secreting EBV+ |
| RPM1 1788              | +++        | Peripheral blood, IgM secreting |
| IM-9                   | +++        | Lymphoblast Ig secreting |
| NC-37                  | +++        | Lymphoblast EBV+ |
| Mouse cell lines       | WEHI-231   | –            | B cell lymphoma          |
| A20                    | –          | B cell lymphoma |

**Discussion**

Here we report the molecular cloning, expression, and biological activity of a new member of the TNF ligand costimulating proliferation by BAFF was unaffected, demonstrating that [3H]thymidine uptake was mainly due to B cell proliferation and not to an indirect stimulation of another cell type (data not shown). The observed B cell proliferation in response to BAFF was entirely dependent on the presence of anti-μ antibodies, indicating that BAFF functioned as costimulator of B cell proliferation.

To investigate a possible effect of BAFF on preplasma, germinal center–like B cells (13), purified peripheral or cord blood B cells were preactivated by coculture with EL-4 T cells in the presence of a cytokine mixture from supernatants of PHA/PMA-stimulated T cells (23). These B cells were resorted to 98% purity and yielded a twofold increase in secreted Ig during a secondary culture in the presence of BAFF and activated T cell cytokines compared with cytokines alone. No significant effect was seen in the absence of exogenous cytokines, and an intermediate (1.5-fold) effect was observed in the presence of the recombinant cytokines IL-2, IL-4, and IL-10 (Fig. 5, E and F).
family. The human and mouse sequences exhibit the typical characteristics of this family, i.e., a type II membrane protein organization and the conservation of nine β sheets, which fold into a “jelly-roll” structure that trimerizes to form receptor interacting sites. The biochemical analysis of BAFF is also consistent with the typical homotrimeric structure of TNF family members. In this family of ligands, BAFF exhibits the highest level of sequence similarity with APRIL, which we have recently characterized as a ligand stimulating growth of various tumor cells (11). Unlike TNF and LTα, which are two family members with equally high homology (33% identity) and whose genes are linked on chromosome 6, APRIL and BAFF are not clustered on the same chromosome. APRIL is located on chromosome 17 (our unpublished data), whereas BAFF maps to the distal arm of human chromosome 13 (13q34). Abnormalities in this locus were characterized in Burkitt lymphomas as the second most frequent defect (24) besides the translocation involving the myc gene into the Ig locus (25). Considering the high expression levels of BAFF-R on all Burkitt lymphoma cell lines analyzed (see Table I), this raises the intriguing possibility that some Burkitt lymphomas may have deregulated BAFF expression, thus stimulating growth in an autocrine manner.

B cell growth was efficiently costimulated with recombinant sBAFF lacking the transmembrane domain. This activity is in contrast to several TNF family members that are active only as membrane-bound ligands, such as TRAIL, FasL, and CD40L. Soluble forms of these ligands have poor biological activity that can be enhanced by their cross-linking, thereby mimicking the membrane-bound ligand (15). In contrast, cross-linking Flag-tagged sBAFF with anti-Flag antibodies or the use of membrane-bound BAFF expressed on the surface of epithelial cells did not further enhance the mitogenic activity of BAFF, suggesting that it can act systemically as a secreted cytokine, like TNF does. This is in agreement with the observation that a polybasic sequence present in the stalk of BAFF acted as a substrate for a pro-
tease. Similar polybasic sequences are also present at corresponding locations in both APRIL and TWEAK (Apo-3L), and for both of them there is evidence of proteolytic processing (26; Holler, N., and J. Tschopp, unpublished observation). Although the protease responsible for the cleavage remains to be determined, it is unlikely to be the metalloproteinase responsible for the release of membrane-bound TNF, as their sequence preferences differ completely (21). The multibasic motifs in BAFF (R-N-K-R), APRIL (R-K-R-R), and TWEAK (R-P-R-R) are reminiscent of the minimal cleavage signal for furin (R-X-K/R-R-R), the prototype of a proprotein convertase family (27).

The role of antigen-specific B lymphocytes during the different stages of the immune response is highly dependent on signals and contacts from helper T cells (28) and antigen-presenting cells such as dendritic cells (29). B lymphocytes first receive these signals early on during the immune response when they interact with T cells at the edge of the B cell follicles in lymphoid tissues, leading to their proliferation and differentiation into low-affinity antibody-forming cells (30). At the same time, some antigen-specific B cells also migrate to the B cell follicle and contribute to the formation of germinal centers, another site of B cell proliferation but also affinity maturation and generation of memory B cells and high-affinity plasma cells (31).

Signals triggered by CD40L have been shown to be critical for the function of B lymphocytes at multiple steps of the T cell–dependent immune response (32). However, several studies clearly showed that CD40L–CD40 interaction does not account for all contact-dependent T cell help for B cells. Indeed, CD40L–deficient T cells isolated from either knockout mice or patients with X-linked hyper IgM syndrome have been shown to successfully induce proliferation of B cells and their differentiation into plasma cells (33). Likewise, studies using blocking antibodies against CD40L showed that a subset of surface IgD+B cells isolated from human tonsils proliferate and differentiate in response to activated T cells in a CD40-independent manner (34). Other members of the TNF family, such as membrane-bound TNF and CD30L, have also been shown to be involved in a CD40- and surface IgM-independent stimulation of B cells (33, 35). Finally, CD40-deficient B cells can be stimulated to proliferate and differentiate into plasma cells by helper T cells as long as the surface B cell receptors are triggered at the same time (36). BAFF as well as CD30L and CD40L is expressed by T cells, but its uniqueness resides in its expression by dendritic cells as well as the highly specific location of its receptor on B cells in contrast to the wider expression patterns of CD40, CD30, and the TNF receptors (37). Hence, BAFF may uniquely affect B cells.

In support of a role for BAFF in T cell– and/or dendritic cell–induced B cell growth and potential maturation, we found that BAFF costimulates proliferation of blood-derived B cells concomitantly with cross-linking of the B cell receptors. Moreover, using CD19+B cells differentiated in vitro into preplasma, germinal center–like B cells (13), we observed a costimulatory effect of BAFF on Ig production by these B cells in the presence of cytokines from activated T cells. Thus, BAFF can induce signals in both naive B cells and germinal center–committed B cells in vivo. Whether this observation will translate during a normal immune response or not will have to be addressed by proper in vivo experiments.

The biological responses induced in B cells by BAFF are distinct from that of CD40L, since proliferation triggered by CD40L occurred at a lower level independently of an anti-μ costimulus (17; Fig. 5 D). Moreover, CD40L can counteract apoptotic signals in B cells after engagement of the B cell receptor (38), whereas BAFF was not able to rescue the B cell line Ramos from the anti-μ-mediated apoptosis, despite the fact that Ramos cells do express BAFF-R (Table I; MacKay, F., unpublished observations). Therefore, it is likely that CD40L and BAFF fulfill distinct functions. In this respect, it is noteworthy that BAFF did not interact with any of 16 recombinant receptors of the TNF family tested, including CD40 (Schneider, P., and J. Tschopp, unpublished observations).

Several obscure zones remain in our understanding of an immune response. For instance, little is known about the mechanisms governing the differentiation of a B cell into a plasma cell versus a germinal center B cell. Similarly, aside from the possible involvement of the CD40 pathway shown in vitro (39), we have very little information about the signals deciding the differentiation of a germinal center B cell into a memory B cell or a plasma cell. It will be very interesting to investigate whether or not BAFF has any unique role to play in these critical checkpoint decisions.
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