Interaction of the TNF homologues BLyS and APRIL with the TNF receptor homologues BCMA and TACI

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BLyS (also called TALL-1, THANK, or BAFF) [1–4] is a member of the tumor necrosis factor (TNF) gene family that stimulates proliferation and immunoglobulin production by B cells. BLyS interacts with the TNF receptor (TNFR) homologue TACI (transmembrane activator and CAML-interactor) [5], and treatment of mice with a TACI–Fc fusion protein abolishes germinal center formation after antigenic challenge [6]. Here we report a novel interaction between BLyS and another TNFR homologue, BCMA (B cell maturation antigen) [7,8]. Further, the TNF homologue APRIL [9], a close relative of BLyS, also bound to BCMA and TACI. BLyS or APRIL activated nuclear factor-κB (NF-κB) through TACI and BCMA, and each ligand stimulated immunoglobulin M (IgM) production by peripheral blood B cells. These results define a dual ligand–receptor system that may play an important role in humoral immunity.

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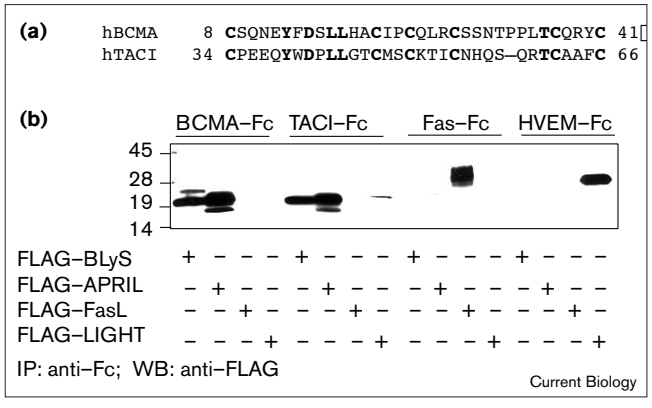
Results and discussion

We have shown previously that BLyS binds to TACI and stimulates NF-κB activity [6]. Unlike most members of the TNFR gene family, which have 3 to 6 cysteine-rich domains (CRDs) in the extracellular region, TACI has two apparent CRDs [5]. One other TNFR homologue, BCMA, appears to have only one CRD. Alignment of the amino acid sequence of this CRD with the amino terminal CRD of TACI revealed 11/33 identities (33%) (Figure 1a). Given this similarity, we investigated whether BLyS could interact with BCMA. We generated a soluble BCMA–Fc fusion protein, and tested its ability to bind to FLAG-epitope-tagged soluble BLyS in a co-immunoprecipitation assay (Figure 1b). FLAG–BLyS bound to BCMA–Fc and to TACI–Fc , but not to Fc fusions of the TNFR homologues Fas [10], or HVEM [11]. In contrast, the TNF homologues FasL [12] and LIGHT [13] bound as expected to Fas–Fc and HVEM–Fc, respectively, but not to BCMA–Fc or TACI–Fc .

The closest structural homologue of BLyS (33% protein sequence identity) is the TNF family member APRIL [9]. Given this similarity, we tested whether APRIL interacts with BCMA or TACI (Figure 1b). Like BLyS, soluble FLAG-tagged APRIL bound to BCMA–Fc and to TACI–Fc . In contrast to a recent report [14], we did not observe binding of FLAG–APRIL to Fas–Fc or HVEM–Fc (Figure 1b). Immunoprecipitation of each ligand through the FLAG tag followed by immunodetection of bound receptor through the Fc tag revealed identical interactions (data not shown).

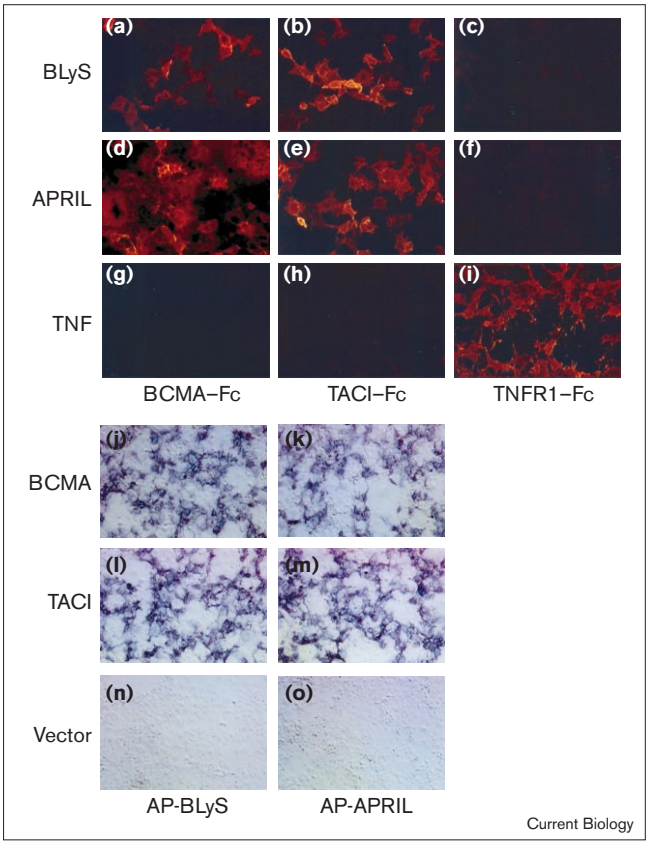
Most TNF family members, including BLyS and APRIL, are expressed as type 2 transmembrane proteins. To test whether the transmembrane forms of these two ligands recognize BCMA and TACI, we transfected COS7 cells with expression vectors carrying each full-length ligand's cDNA, and stained the cells with receptor–Fc fusion proteins. BCMA–Fc and TACI–Fc bound to cells transfected

Figure 1



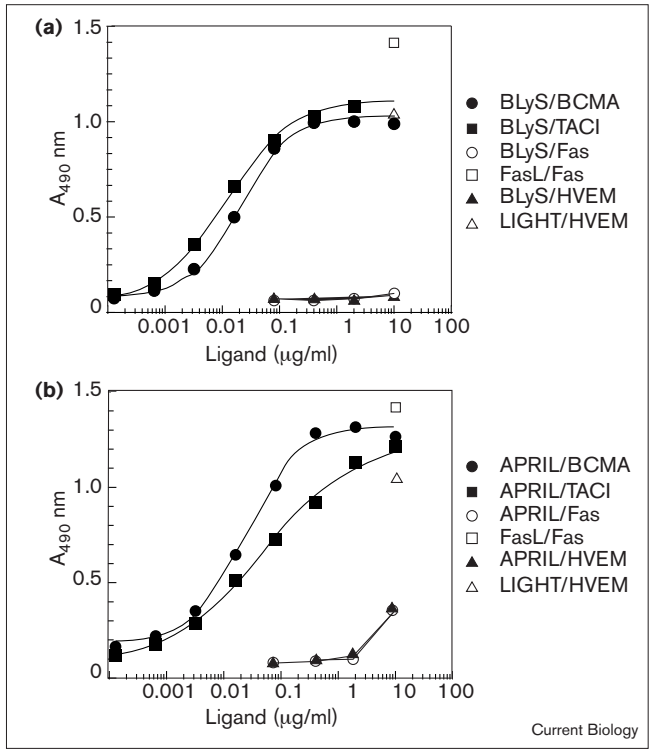
(a) Comparison of the amino acid sequences of the amino-terminal CRDs of BCMA and TACI. Identities are shown in bold. **(b)** Interaction of BLyS and APRIL with BCMA and TACI. Purified BCMA–Fc, TACI–Fc, Fas–Fc, or HVEM–Fc (1 μg/ml) was incubated with purified FLAG–BLyS, FLAG–APRIL, FLAG–FasL, or FLAG–LIGHT (1 μg/ml) as indicated for 1 h at 24°C. The reactions were subjected to immunoprecipitation (IP) through the receptor–Fc fusion with protein-A-agarose and analyzed by western blot (WB) with an anti-FLAG antibody M2 (Sigma) to detect the bound ligands.

Figure 2



Interaction of soluble receptors or ligands with cells transfected with the corresponding binding partners. **(a–i)** COS7 cells were transfected with expression plasmids encoding full-length (a–c) BLyS, (d–f) APRIL, or (g–i) TNF [20], and 24 h later incubated with BCMA–Fc, TACI–Fc, or TNFR1–Fc [21] as indicated for 1 h at 37°C. The cells were washed, fixed, and the binding of Fc-fusion protein was detected by biotinylated goat anti-human Fc antibody followed by Cy3–streptavidin. **(j–o)** COS 7 cells were transfected with expression plasmids encoding (j,k) native sequence full-length BCMA or (l,m) TACI, or (n,o) with vector, and 24 h later incubated with conditioned medium from 293 cells transfected with AP–BLyS or AP–APRIL as indicated for 1 h at 37°C. The cells were washed, fixed, and stained *in situ* for AP activity.

Figure 3



Binding curves for ligand–receptor interactions. Fc-fusion proteins of BCMA, TACI, Fas, or HVEM were immobilized on microtiter wells coated with goat-anti-human Fc antibody, and incubated with varying concentrations of purified FLAG-tagged BLyS **(a)**, APRIL **(b)**, FasL or LIGHT (a,b) as indicated for 1 h at 24°C. The binding was determined with biotinylated anti-FLAG M2 antibody followed by colorimetric substrate conversion with horseradish peroxidase-conjugated streptavidin. The plots are from one of three to four representative experiments, from which mean ± SE of the apparent dissociation constant (K_D) were calculated (see text).

with transmembrane BLyS or APRIL but not to cells transfected with full-length TNF, whereas TNFR1–Fc bound to cells transfected with TNF, but not to BLyS- or APRIL-transfected cells (Figure 2a–i).

Unlike the majority of known TNFR family members [15], but similar to TACI [5], BCMA does not contain a typical amino-terminal signal sequence, suggesting that it may be a type 3 rather than a type 1 transmembrane protein. Previous work suggested that BCMA is located in the Golgi compartment [16]. If BCMA and TACI are expressed at the cell surface, then they should be recognized in that location by BLyS and APRIL. To test this notion, we transfected COS7 cells with expression vectors encoding each native-sequence, full-length receptor, and stained the cells with soluble alkaline-phosphatase (AP) fusion proteins of each ligand (Figure 2j–o). Both AP–BLyS and AP–APRIL bound to cells transfected with BCMA or TACI but not to vector-transfected controls. Thus, BCMA and TACI can localize to the cell surface, where they can be identified by each ligand.

To assess the affinity of these interactions, we measured the dosage dependence of ligand binding to each receptor–Fc fusion protein (Figure 3). BLyS exhibited a slightly higher affinity for TACI–Fc (K_D = 160 ± 30 pM) as compared to BCMA–Fc (K_D = 260 ± 10 pM; Figure 3a). APRIL, on the other hand, showed a somewhat higher affinity for BCMA–Fc (K_D = 500 ± 100 pM) than for TACI–Fc (K_D = 910 ± 200 pM; Figure 3b). Whereas FasL and LIGHT showed a high level of binding, respectively, to Fas–Fc and HVEM–Fc, BLyS showed no binding and APRIL showed marginal binding (Figure 3). Thus, the

binding of BLyS or APRIL to BCMA or TACI occurs at apparently physiological concentrations.

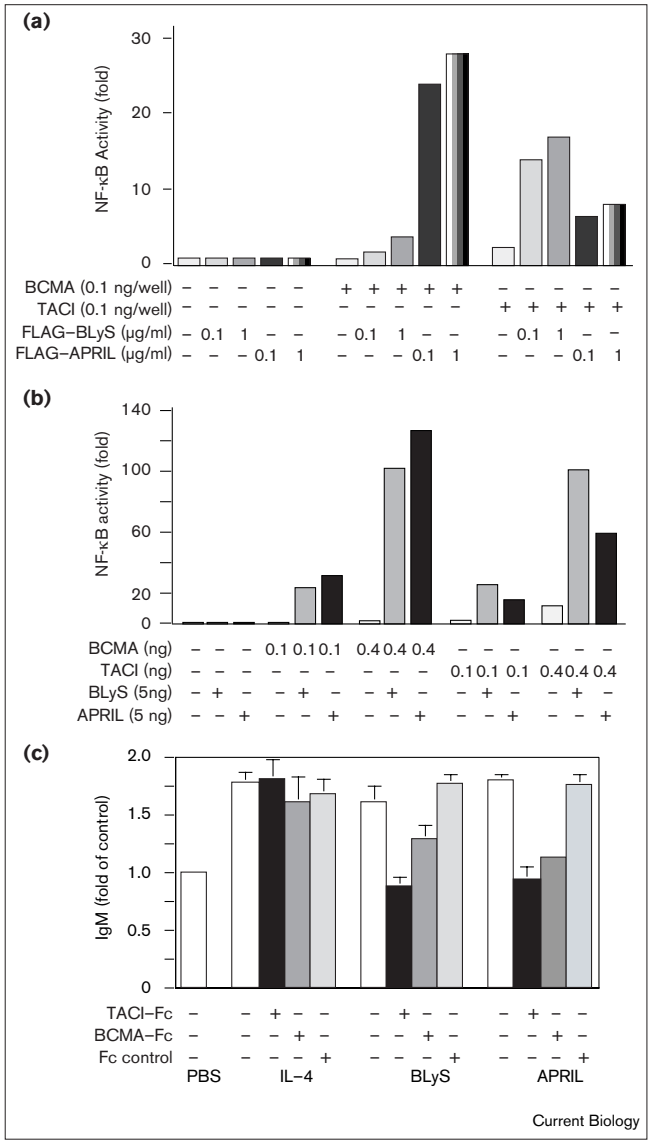
The signaling mechanisms of BCMA have not been characterized. Therefore, we investigated whether BCMA activates NF-κB in response to BLyS (Figure 4a,b). In addition, we tested APRIL's ability to stimulate NF-κB through BCMA or TACI. Treatment of 293 cells transfected with expression vectors encoding native sequence, full-length BCMA or TACI with purified soluble FLAG-tagged BLyS or APRIL induced marked NF-κB activation relative to controls, as measured by a reporter-gene assay (Figure 4a). Next, we examined whether co-transfection of 293 cells with expression plasmids encoding each full-length ligand and receptor combination leads to NF-κB stimulation (Figure 4b). BLyS and APRIL induced significant NF-κB activation through BCMA and TACI. Thus, both BLyS and APRIL can stimulate NF-κB through either BCMA or TACI. TACI has been reported to activate the T cell transcription factor NF-AT [5]. Because BCMA and TACI activated NF-κB despite the lack of clear sequence homology in their cytoplasmic region, it is formally possible that BCMA can activate NF-AT as well.

BLyS is implicated in B-cell activation and it stimulates immunoglobulin production by B cells [1–4,17]. We investigated whether APRIL modulates B cells as well by testing B cell IgM production in cultures of peripheral blood leukocytes (Figure 4c). Both BLyS and APRIL induced a marked enhancement of IgM production, similar to interleukin-4 (IL-4). TACI–Fc and BCMA–Fc inhibited the effect of BLyS and APRIL but not of IL-4. Combination of BLyS and APRIL did not result in further stimulation (data not shown), consistent with the notion that the two ligands use the same receptors to modulate B cells.

Our results demonstrate that BLyS is not the sole ligand of TACI and BCMA; rather, the closest relative of BLyS in the TNF gene family, APRIL, interacts with these same receptors. APRIL has been reported to stimulate the growth of various tumor cell lines [9]; however, its physiological role is not fully understood. Whether TACI, BCMA, or some other receptor(s) mediate APRIL-induced proliferation is yet to be investigated.

In previous work, we identified TACI as a B-cell receptor for BLyS by expression cloning [6]. Treatment of mice with a TACI–Fc fusion protein after antigenic challenge diminished IgM and IgG production and abolished splenic germinal center formation. These data suggested that the interactions of BLyS and TACI are crucial for humoral immunity. During review of this manuscript, Gross *et al.* [18] reported that TACI and BCMA are receptors for BLyS and showed that treatment with TACI–Fc ameliorated disease and improved survival in mouse models of systemic lupus erythematosus (SLE), a B-cell-mediated autoimmune disease. Taken together, these findings suggest that TACI–Fc exerts its therapeutic effect on mouse SLE by perturbing the generation of

Figure 4



Activation of NF-κB and of IgM production by BLyS and APRIL. **(a)** Human 293 cells were transfected with the indicated amounts of expression plasmids encoding native sequence full-length BCMA or TACI along with 0.25 μg of ELAM-luciferase reporter gene plasmid, and 25 ng pRL-TK. After 4 h, purified soluble FLAG-tagged BLyS or APRIL was added at the indicated concentration for 20 h, and reporter gene activity determined with the dual-luciferase reporter assay system (Promega). **(b)** Human 293 cells were co-transfected with the indicated amounts of expression plasmids for each ligand-receptor combination and NF-κB activity was determined as above. **(c)** Human peripheral blood leukocytes were incubated for 72 h with PBS, or IL-4 (100 ng/ml), or FLAG–BLyS or FLAG–APRIL (1 μg/ml), alone, or in combination with 20 μg/ml TACI–Fc, BCMA–Fc, or a human IgG1 control. Cell supernatants were analyzed for IgM levels by ELISA (Bethyl Laboratories). The data are means ± SEM of four experiments.

autoantibodies. Although these effects of TACI–Fc were previously ascribed to inhibition of BLyS [6,18], this study, which uncovers APRIL as a second ligand for TACI, suggests that APRIL inhibition also may contribute to the effects of TACI–Fc on immunization and SLE. In addition, it is likely that TACI–Fc blocks the interactions of BLyS and APRIL not only with cellular TACI, but also with BCMA, which is expressed in lymphoid organs and B lymphocytes [19]. Thus, BLyS and APRIL and their receptors TACI and BCMA may act in concert to regulate B-cell function. Further studies, particularly gene knockout experiments, should help define more fully the individual contribution of each member of this newly defined ligand–receptor system to humoral immunity in health and disease.

Materials and methods

Fc fusion proteins (immunoadhesins) were constructed using amino acids 5–51 of human BCMA [7,8], 2–166 of human TACI [5], 1–170 of Fas [10], or 1–199 of HVEM [11], and expressed in Chinese hamster ovary (CHO) cells using a heterologous signal sequence (prepro-trypsin amino acids 1–17) for BCMA and TACI, or the endogenous signal sequence for Fas and HVEM. FLAG-tagged ligands were constructed in pCMV-1 Flag (Sigma) using amino acids 124–285 of human BLyS [1], 105–250 of human APRIL [9], 103–251 of human FasL, and 82–240 of human LIGHT [13]. Ligands were expressed in CHO cells and purified by affinity chromatography on anti-Flag M2-agarose (Sigma).

AP fusion proteins were constructed by inserting the human AP coding sequence derived from pAPtag-5 (Genehunter Corp.) into each pCMV-1 FLAG–ligand vector between the Flag tag and the ligand sequences.

The total amount of transfected DNA in co-transfection experiments was kept constant at 1 μg by supplementation with empty vector.

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