TNFR-Associated Factor-3 Is Associated With BAFF-R and Negatively Regulates BAFF-R-Mediated NF-κB Activation and IL-10 Production

Liang-Guo Xu and Hong-Bing Shu

*J Immunol* 2002; 169:6883-6889; doi: 10.4049/jimmunol.169.12.6883

http://www.jimmunol.org/content/169/12/6883

References

This article cites 59 articles, 26 of which you can access for free at:

http://www.jimmunol.org/content/169/12/6883.full#ref-list-1

**Why The JI?** Submit online.

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
TNFR-Associated Factor-3 Is Associated With BAFF-R and Negatively Regulates BAFF-R-Mediated NF-κB Activation and IL-10 Production

Liang-Guo Xu* and Hong-Bing Shu2*†

TALL-1, also called BAFF, Blys, THANK, and zTNF4, is a member of the TNF family of ligands identified by us and others (1–5). TALL-1 is specifically and constitutively expressed by monocytes, macrophages, and dendritic cells (1–3, 6), and is induced by IFN-γ and IL-10 (3, 6). Like most members of the TNF family, the extracellular domain of TALL-1 can be cleaved to form a soluble cytokine (2). Crystal structure studies suggest that soluble TALL-1 (sTALL-1) contains an unique “flap” region that is important for its virus-like assembly, receptor binding, and biological activities (7–9).

TALL-1 signals through three receptors, including BCMA, TACI, and BAFF-R, which are members of the TNFR family (5, 10–24). All three receptors are mainly expressed by B lymphocytes, while TACI is also induced in a subset of T cells following their activation (17–24). Early studies suggest that sTALL-1 can potently stimulate B lymphocyte proliferation in vitro, either alone or in synergy with anti-IgM (2, 3, 10–13). It is now believed that sTALL-1 promotes either differentiation of B cells or survival of mature B cells (25–28). Administration of recombinant sTALL-1 or overexpression of sTALL-1 in mice leads to increased numbers of mature B lymphocytes, splenomegaly, anti-DNA Abs, proteinuria, and glomerulonephritis, phenotypes that mimic those of systemic lupus erythematosus (3, 5, 14, 29, 30). Conversely, it has been shown that recombinant soluble TACI-Ig fusion proteins can significantly inhibit progression of lupus-like autoimmune syndrome and collagen-induced arthritis in animal models (27, 31). Recently, gene knockout studies further confirmed that TALL-1 is required for normal B cell development (26). Surprisingly, gene inactivation studies also indicated that BAFF-R, but not TACI and BCMA, is required for TALL-1-triggered B cell development (26, 32–34). Taken together, these studies suggest that the TALL-1/BAFF-R signaling plays critical roles in regulation of B cell function and autoimmune diseases.

The signal transduction pathways triggered by BCMA, TACI, and BAFF-R are poorly characterized. Like many other members of the TNFR family, BCMA and TACI can bind to TNFR-associated factor (TRAF) proteins and activate the transcription factor NF-κB and the serine/threonine protein kinase JNK (10–13, 16, 35). In addition, it has also been shown that TACI can activate the transcription factor NF-AT (36).

The intracellular signaling pathways and downstream effectors triggered by BAFF-R are not known. In this study, we identified TRAF3 as a BAFF-R-associated protein. Our findings suggest that TRAF3 negatively regulates BAFF-R-mediated NF-κB activation and IL-10 production.

Materials and Methods

Reagents and cell culture

Mammalian-derived recombinant Flag-tagged human sTALL-1 (10) and Escherichia coli-derived recombinant His6-tagged human sTALL-1 (7) were previously described. The human embryonic kidney 293 (Dr. Z. Cao, Tulark, South San Francisco, CA), the B lymphoma Bjab (Dr. J. Hagman, National Jewish Medical and Research Center, Denver, CO) and RPMI 8226 (American Type Culture Collection (ATCC), Manassas, VA) cells, the mAbs against Flag (Sigma-Aldrich, St. Louis, MO) and hemagglutinin (HA) (Covance, Berkeley, CA) epitopes, and the rabbit polyclonal anti-TRAF3 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) were obtained from the indicated sources.

*Department of Immunology, National Jewish Medical and Research Center, University of Colorado Health Sciences Center, Denver, CO 80206; and †Department of Cell Biology and Genetics, College of Life Sciences, Peking University, Beijing, People’s Republic of China

Received for publication July 12, 2002. Accepted for publication October 11, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C., Section 1734 solely to indicate this fact.

This work was supported by grants from the Ellison Medical Foundation, National Institutes of Health (1RO1 AI09992-01), Arthritis Foundation, National Natural Science Foundation of China (39925016), Chinese “863” Program (2001AA221281), and Special Funds for Major State Basic Research of China (G19990539).

Address correspondence and reprint requests to Dr. Hong-Bing Shu, Department of Immunology, National Jewish Medical and Research Center, 1400 Jackson Street, K516c, Denver, CO 80206. E-mail address: shuh@njc.org

Abbreviations used in this paper: sTALL-1, soluble TALL-1; T2, transitional type II; LTβR, lymphotxin-β receptor; TRAF, TNFR-associated factor; HA, hemagglutinin.

Copyright © 2002 by The American Association of Immunologists, Inc. 0022-1767/02/$02.00
TRAF3 is involved in BAFF-R signaling

293 cells (5 x 10⁶) were transfected with the indicated plasmids. Twenty-four hours after transfection, cells were lysed in 1 ml lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton, 1 mM EDTA, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PMSF). For each immunoprecipitation, 0.4 µl affinity-purified antibody or mouse IgG was added to the indicated cell lysates, which were then incubated for 4 h at 4°C. The precipitates were fractionated on SDS-PAGE and subsequently Western blot analyses were performed as described (37, 38).

Vectors

To construct C-terminal HA-tagged expression plasmids for BCMA, TACI, BAFF-R, and BAFF-R mutants, cDNAs for these proteins were amplified by PCR from a B cell cDNA library and inserted into a C-terminal HA-tagged pcDNA3 plasmid (39).

To make retroviral vectors for TRAF3 and its TRAF domain (aa 258–568) mutant (TRAF3-C), cDNAs encoding for full-length TRAF3 and aa 258–568 were amplified by PCR and inserted into the MSCV2.2IRES–GFPα retroviral vector (provided by Dr. P. Marrack, National Jewish Medical and Research Center).

Expression plasmids for TRAF1, 2, 3, 5, 6 (Dr. D. Goeddel, Tularik) and NF-κB-luciferase reporter plasmid (Dr. G. Johnson, University of Colorado Health Sciences Center, Denver, CO) were provided by the indicated investigators.

Establishment of BCMA, TACI, and BAFF-R stable cell lines

Expression plasmids for C-terminal HA-tagged BCMA, TACI, and BAFF-R were linearized and transfected into RPMI 8226 cells or Bjab cells by electroporation. The transfected cells were selected by G418 (1 mg/ml) for 2 wk. Cells with high-level receptor expression were sorted with Flag-tagged sTALL-1 by flow cytometry. Overexpression of the receptors in the stable lines were confirmed by Western blot with anti-HA Ab.

Establishment of TRAF3 and TRAF3-C-terminal stable cell lines

Retroviral plasmid (15 µg) for wild-type TRAF3 or TRAF3-C was transfected into the packaging cell line 293–10A1 (~2 x 10⁶) by calcium phosphate precipitation. Eighteen hours after transfection, the cells were washed with PBS and cultured in 5 ml of fresh medium for 24 h. The supernatant was added to retrovirus-containing medium that was collected and centrifuged. The supernatant, supplemented with 4 µg/ml of polybrene, was used to infect RPMI 8226 or Bjab cells (~2 x 10⁶). Two days after infection, green fluorescent protein-positive cells were isolated using a cell sorter.

Flow cytometry analysis

RPMI 8226 or Bjab cells were incubated in staining buffer (PBS/2% FBS) in the absence or presence of Flag-sTALL-1 (100 ng/ml) for 40 min. Cell staining was performed by sequential incubation (each 40 min) with anti-Flag mAb (1 µg/ml) and R-PE-conjugated goat anti-mouse IgG (1/200 dilution) in staining buffer. Cells were washed twice with staining buffer following each incubation. Cells with high-level Flag-sTALL-1 binding were isolated by a cell sorter.

Reporter gene assays

Bjab stable cell lines (~2 x 10⁵) were seeded on 6-well (35-mm) dishes and were transfected with 1.0 µg of NF-κB-luciferase reporter plasmid by Lipofectamine 2000 (Invitrogen, Carlsbad, CA). To normalize for transfection efficiency and protein amount, 0.5 µg of RSV-β-galactosidase plasmid was added to all transfections. Fourteen hours after transfection, cells were treated with His-sTALL-1 (200 ng/ml) or untreated for 6 h. Luciferase reporter assays were performed using a luciferase assay kit (BD PharMingen, San Diego, CA) and following the manufacturer’s protocols. β-Galactosidase activity was measured using the Galacto-Light chemiluminescent kit (Tropix, Medford, MA). Luciferase activities were normalized on the basis of β-galactosidase expression levels.

ELISA

Human IL-10 ELISA were performed using the human IL-10 ELISA Ready-Set-Go kit (eBioscience, San Diego, CA) by following procedures recommended by the manufacturer.

Results

Identification of TRAF3 as a specific BAFF-R-interacting protein

The intracellular signaling pathways mediated by BAFF-R are unknown. Similar to other members of the TNFR family, BAFF-R does not have intrinsic enzymatic activity and is believed to transduce signals through physical interaction with downstream signaling proteins. To identify BAFF-R-associated signaling proteins, we used the yeast two-hybrid system to screen a human B cell cDNA library with the intracellular domain of BAFF-R as bait. We screened a total of 5 million independent clones and obtained 78 β-galactosidase-positive clones. Sequence analysis of 20 clones indicated that most clones encode a poorly characterized protein pl45 (40). Subsequent transient transfection and coimmunoprecipitation experiments indicated that BAFF-R did not interact with pl45 in 293 cells (data not shown). One of the sequenced clones encodes for TRAF3.

Because TRAF proteins are involved in signaling by many members of the TNFR family (41–43), we decided to investigate a potential role for TRAF3 in BAFF-R signaling. To determine whether TRAF3 is associated with BAFF-R in mammalian cells, expression plasmids for C-terminal HA-tagged BAFF-R and N-terminal Flag-tagged TRAF3 were transfected into 293 cells. Coimmunoprecipitation experiments indicated that BAFF-R interacted with TRAF3 (Fig. 1A). In the same experiments, BAFF-R did not interact with other cytoplasmic TRAF proteins, including TRAF1, TRAF2, TRAF5, and TRAF6 (Fig. 1A). TRAF4 was not included in these experiments because it is primarily localized in the nucleus in mammalian cells (41–43). These data suggest that BAFF-R specially interacts with TRAF3.

Domain mapping of the interaction between BAFF-R and TRAF3

TRAF3 proteins interact with members of the TNFR family through their TRAF domains (41–43). We determined whether the TRAF domain of TRAF3 (TRAF3-C) is sufficient to interact with BAFF-R. As shown in Fig. 1B, transient transfection and coimmunoprecipitation experiments suggest that the TRAF domain of TRAF3 is sufficient for interaction with BAFF-R.

Previously, conserved TRAF-binding motifs, such as (P/S/A/T)x(Q/E)x and PxQxxD, have been identified in the cytoplasmic domains of some TNFR family members (41–44). However, these conserved TRAF-binding motifs are not easily recognizable in the cytoplasmic domain of BAFF-R. To determine the regions of BAFF-R that are required for interaction with TRAF3, we constructed a series of C-terminal HA-tagged deletion mutants of the BAFF-R (Fig. 2A). Transient transfection and coimmunoprecipitation experiments suggest that six aa (PDGDKD) at the membrane proximal region (aa 117–122) and the C-terminal tail (aa 150–184) are both required for interaction with TRAF3 (Fig. 2, A and B).

TRAF3 is recruited to BAFF-R by sTALL-1 stimulation

Under physiological conditions, BAFF-R is specifically expressed in B lymphocytes. We next determined whether BAFF-R interacts with TRAF3 in B lymphoma cells and whether this interaction is affected by sTALL-1 stimulation.
enhanced the interaction between BAFF-R and TRAF3. The weak interaction between BAFF-R and TRAF3 in the absence of sTALL-1 is probably due to the fact that overexpression of BAFF-R mimics TALL-1 stimulation of endogenous BAFF-R. It is possible that the association of TRAF3 with BAFF-R is dependent on TALL-1 stimulation in untransfected cells. In these experiments, TRAF3 was also recruited to TACI, but not to BCMA, in a ligand-dependent manner (Fig. 3). These data are consistent with previous observations that TRAF3 interacts with TACI, but not BCMA, in mammalian overexpression systems (10, 13).

Inhibition of BAFF-R-mediated NF-κB activation by TRAF3

Many TNFR family members, including TALL-1 receptors BCMA and TACI, can activate the transcription factor NF-κB. Previously, we have shown that TALL-1 can induce NF-κB activation in the B lymphoma RPMI 8226 cells (39). To determine whether BAFF-R can activate NF-κB, we transfected a NF-κB-luciferase reporter plasmid into Bjab cells overexpressing BAFF-R. Luciferase assays indicated that overexpression of BAFF-R could significantly induce NF-κB activation, and this effect was enhanced by sTALL-1 stimulation (Fig. 4). Moreover, overexpression of TRAF3 and TRAF3-C both inhibited BAFF-R-mediated NF-κB activation (Fig. 4).

Inhibition of BAFF-R-mediated IL-10 expression by TRAF3

Previously, we showed that TALL-1 stimulation or overexpression of BAFF-R in RPMI 8226 cells induced IL-10 production (39). To determine whether TRAF3 is involved in BAFF-R-induced IL-10 production, we transfected TRAF3 or its TRAF domain into BAFF-R-overexpressing RPMI 8226 cells by retroviral-mediated gene transfer and measured IL-10 levels by ELISA. The results indicated that both wild-type TRAF3 and TRAF3-C inhibited BAFF-R-induced IL-10 production (Fig. 5).

Discussion

After migrating from the bone marrow to the spleen, immature B cells undergo two transitional stages, transitional type I and II (T2), before differentiating into naive mature B cells. In vitro experiments suggest that sTALL-1 specifically promotes the survival of T2 B cells and their differentiation into mature B cells (25, 27, 28). Consistent with these observations, inhibition of TALL-1 signaling by administration of TACI-Ig decoy receptor or gene knockout of TALL-1 causes deficiency of T2 and mature B cells (26, 27, 31). These studies point to a crucial role for TALL-1 in B cell survival and maturation. Among the three TALL-1 receptors, BCMA and TACI are not important for B cell development because normal B cell maturation is found in BCMA- and TACI-deficient mice (32–34). In contrast, inactivation of BAFF-R in mice causes loss of T2 and mature B cells (26). These studies suggest that BAFF-R signaling is essential for TALL-1-triggered B cell survival and maturation.

The downstream effector molecules responsible for TALL-1/BAFF-R-triggered B cell survival and maturation are not clear. It is possible that TALL-1 signaling initiates the anti-apoptotic activity that is associated with the activation of NF-κB. Previously, it has been shown that disruption of NF-κB activation pathways, such as by gene knockout of IKKα, Rel, and RelA, impairs B cell maturation (45–47). In this context, we found in this study that BAFF-R could also activate NF-κB.

Both BCMA and TACI can activate NF-κB but are not important for TALL-1-triggered B cell survival, suggesting that activation of NF-κB is not sufficient for TALL-1-triggered B cell survival. Recently, we identified multiple downstream genes transcriptionally induced by TALL-1, including the cytokine IL-10, the chemokine...
LAG-1, and GCP-2, the secreted protein pre-B cell colony enhancing factor, among others (39). Among the genes induced by TALL-1, IL-10 is particularly interesting. It has been shown that IL-10 can suppress cytokine production and several accessory cell functions by Th1 cells, macrophages, and NK cells and is regarded as a potent suppressor of the effector functions of these cells (48). Conversely, IL-10 is a potent stimulator of B cell proliferation and differentiation and is critically involved in regulating autoantibody-secreting B cell activities in systemic lupus erythem (49, 50). Our previous studies suggest that BAFF-R, but not TACI and BCMA, can dramatically up-regulate IL-10 expression in primary and transformed B cells (39), pointing to the possibility that IL-10 is an important effector molecule for TALL-1-triggered B cell survival and maturation.

In an attempt to decipher the intracellular signaling pathways mediated by BAFF-R, we identified TRAF3 as a cytoplasmic protein physically binding to the cytoplasmic domain of BAFF-R. TRAF3 is a member of the TRAF protein family, which is involved in signaling by many members of the TNFR family (41–43). TRAF3 was first identified as a molecule that binds to the cytoplasmic tails of CD40 and EBV-transforming protein LMP1 (51–54). Signaling through CD40 in B cells causes rescue from apoptosis, proliferation, differentiation, Ig production, class switching, and expression of costimulatory molecules. Overexpression of TRAF3 inhibits CD40-mediated Ab secretion (54). The TRAF domain of TRAF3 is sufficient to bind to CD40 and also inhibits CD40-mediated Ab secretion when overexpressed, suggesting that the physical association of TRAF3 with CD40 mediates its negative regulatory function (55). TRAF3 is also recruited in a ligand-dependent manner to lymphotoxin-β receptor (LTβR) and has an inhibitory effect on LTβR-mediated NF-κB activation (56, 57). In this context, it is interesting that both TRAF3 and its TRAF domain inhibit BAFF-R-mediated downstream effects, such as NF-κB activation and IL-10 production. Taken together, these studies suggest that TRAF3 is involved in negative regulation of
signaling by several TNFR family members, including BAFF-R, CD40, and LTβR.

The cytoplasmic domain of BAFF-R is not conserved with those of other TNFR family members. The major conserved TRAF-binding motif, (P/S/A/T)x(Q/E)E (44), is not easily recognizable in the cytoplasmic domain of BAFF-R. A 6-aa sequence at position 117 of BAFF-R, PDGDKD, is weakly similar to the minor consensus TRAF-binding motif PxQxxD found in the EBV LMP1 protein and the TRAF-interacting protein I-TRAF/TANK (44). Previously, it has been shown that TRAF3 binds to LTβR through amino acids PEEGDPG at position 389 of LTβR (57). This site is also not found in BAFF-R. We made a series of deletion mutants of BAFF-R and examined their interaction with TRAF3 in coimmunoprecipitation experiments. These studies suggest that the PDGDKD motif at position 117 and the C-terminal 35 aa are both required for BAFF-R’s association with TRAF3. The simplest explanation for this observation is that these amino acids form a novel spatial structure for BAFF-R binding to TRAF3. In this context, it has previously been shown by crystal structure studies that binding of TRAF3, but not TRAF2, to CD40 is influenced by both the PXQXT motif and residues distal to this site (58–60). Alternatively, it is also possible that TRAF3 binds only to one site in the cytoplasm of BAFF-R in vivo and the results from our mapping experiments are due to altered folds caused by deletion.

In conclusion, we have identified TRAF3 as a BAFF-R-associated signaling protein. TRAF3 can inhibit BAFF-R-mediated NF-κB activation and IL-10 production, suggesting a negative regulatory role for TRAF3 in TALL-1-triggered B cell survival and maturation.

Acknowledgments

We thank Drs. Zhaodan Cao, David Goeddel, Gary Johnson, and Philippa Marrack for reagents.
References


