Identification of a receptor for BLyS demonstrates a crucial role in humoral immunity

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B lymphocyte stimulator (BLyS) is a member of the tumor necrosis factor (TNF) superfamily. BLyS stimulates proliferation of, and immunoglobulin production by, B cells. However, the relative importance of BLyS in physiological B cell activation is unclear. We identified a B cell receptor for BLyS through expression cloning as TACI, an orphan TNF receptor homologue of unknown function. Binding of BLyS to TACI activated signaling by nuclear factor-κB (NF-κB). *In vitro* soluble TACI-Fc fusion protein blocked BLyS-induced NF-κB activation in B lymphoma cells and IgM production in peripheral blood B cells. *In vivo* treatment of immunized mice with TACI-Fc inhibited production of antigen-specific IgM and IgG1 antibodies and abolished splenic germinal center (GC) formation. Thus, BLyS activity must play a critical role in the humoral immune response.

The tumor necrosis factor (TNF) ligand superfamily consists of more than 15 members that regulate a variety of cellular functions, including proliferation, differentiation and apoptosis[1]. TNF and most of its homologues are Type II transmembrane proteins which can be released by cells as soluble homotrimeric molecules. These ligands interact with specific members of the TNF receptor (TNFR) superfamily, which contains more than 20 proteins[2,3]. Several TNF superfamily ligands, including TNF (TNFSF2), LTβ (TNFSF3), CD40L (TNFSF5) and FasL (TNFSF6), are important modulators of immunity and play essential roles in lymphoid or thymic development, peripheral deletion, T cell-mediated immune responses, T cell-dependent help for B cells, or humoral B cell activity[1].

BLyS (TNSF13B) a newly identified TNF homologue, is implicated in the regulation of B cell function[4–7]. BLyS is expressed on T cells, dendritic cells, monocytes and macrophages[4–7], and binding sites for BLyS are found primarily on B cells[4,7]. In a similar manner to CD40L[1], BLyS promotes B cell proliferation *in vitro* during costimulation by antibody to IgM[7]. Short-term administration of soluble BLyS to mice disrupts the architecture of B and T cell zones in the spleen and elevates serum IgM levels[6]. Transgenic mice expressing the *BLyS* gene systemically show global B cell activation and develop autoimmune disorders[8,9]. These findings suggest that BLyS may be a positive regulator of B cell function, however, the molecular basis of BLyS function and the relative contribution of this ligand to humoral immunity is unknown. To address these questions, we looked for a receptor for BLyS in B cells. Through expression cloning, we identified such a BlyS receptor. It was a TNFR homologue which had no previously known ligand. We found that BLyS stimulation of this receptor *in vitro* led to activation of nuclear factor-κB (NF-κB) and induced immunoglobulin production by B cells. To investigate the importance of BLyS in the development of a primary immune response, we used the TACI receptor's extracellular domain as a soluble inhibitor to block BLyS activity after antigen challenge *in vivo*. Our results indicate that BLyS plays an important role in the induction of immunoglobulin production by B cells and is essential for formation of splenic germinal centers (GCs), the sites of antibody affinity maturation and memory B cell formation.

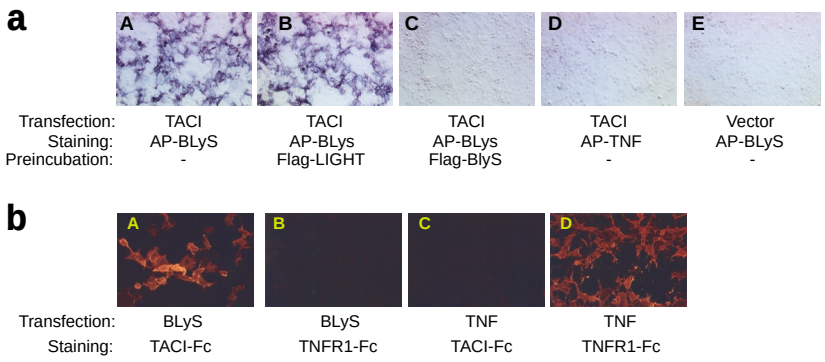

Figure 1. Interaction of TACI with BlyS on cells. (**a**) Monkey COS-7 cells were transfected with human *TACI* (**A–D**) or vector (**E**), incubated with conditioned medium from Chinese hamster ovary cells transfected with human AP-BLyS (**A,B,C,E**) or AP-TNF (**E**), and stained for alkaline phosphatase activity *in situ*. Where indicated, cells were preincubated with 2 μg/ml of purified soluble human Flag-LIGHT or Flag-BLyS. **(b)** COS-7 cells transfected with full-length *BLyS* (**A,B**) or *TNF* **(C,D)** were incubated with 1 μg/ml of human TACI-Fc **(A,C)** or TNFR1-Fc **(B,D)** and stained with biotinylated goat anti-human Fc antibody and Cy3-streptavidin.

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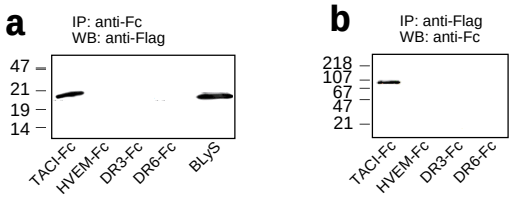

Figure 2. Interaction of TACI and BLyS in solution. Purified human TACI-Fc, HVEM-Fc, DR3-Fc, or DR6-Fc (1 μg/ml) was incubated with purified human Flag-BLyS (1 μg/ml) in duplicate. (**a**) One set of reactions was subjected to immunoprecipitation (IP) through the receptor-Fc fusion with protein-A-agarose; for comparison, the input amount of Flag-BLyS was loaded directly in the right hand lane. (**b**) A second set of reactions was subjected to IP through the ligand with antibody to Flag (anti-Flag). The samples were analyzed by western blot (WB) with either: (**a**) anti-Flag to detect the ligand or (**b**) antibody to human Fc to detect Fc-fused receptors.

Results

TACI is a B cell receptor for BLyS

To identify a B cell receptor for BLyS, we used the expression cloning technique[10]. We generated two tagged versions of the ligand: mature soluble human BLyS (amino acids 136–285)[4,7] fused at the NH_2-terminus to human placental alkaline phosphatase (AP-BLyS); and mature soluble human BLyS fused similarly to a Flag epitope-tag (Flag-BLyS). We constructed a cDNA expression library from the human IM-9 multiple myeloma B cell line; IM-9 cells exhibited a high level of AP-BLyS binding, which could be blocked by preincubation with Flag-BLyS but not with TNF (data not shown). Pools of cDNA clones from the IM-9 library were transfected into monkey COS-7 cells, and screened for AP-BLyS binding by *in situ* alkaline phosphatase activity. After three rounds of screening, one positive cDNA was identified. Sequencing revealed a single open reading frame that encoded an orphan member of the TNFR family called TACI (transmembrane activator and CAML-interactor)[11]. *TACI* was originally isolated from a human B lymphocyte cDNA library, in a yeast two-hybrid screen, by its interaction with an intracellular adaptor termed calcium-modulator and cyclophilin ligand (CAML), which costimulates the transcription factor nuclear factor of activated T cells (NF-AT)[11]. Surface expression of TACI was detected on resting, as well as activated, peripheral B cells, and on activated but not on resting T cells[11]. However, the role of TACI in the immune response was not defined.

Transfection of a human TACI expression plasmid into COS-7 cells conferred strong binding of human AP-BLyS, but not AP-TNF[12] (**Fig. 1a**); AP-BLyS binding to TACI-transfected cells was effectively blocked by Flag-BLyS, but not by LIGHT, another TNF homologue[13]. An Fc fusion protein based on the human TACI ectodomain (TACI-Fc)[11,14] bound to COS-7 cells transfected with a plasmid encoding the full-length transmembrane form of human BLyS, but not to cells transfected with transmembrane human TNF. Conversely, a human TNFR1-Fc fusion protein bound to *TNF*-transfected cells, but not to *BLyS*-transfected cells (**Fig. 1b**). We also found that TACI-Fc did not bind to transfected cells expressing several other human TNF family members in transmembrane form, including CD27L (TNFSF7), CD30L (TNFSF8), CD40L, OX-40L (TNFSF4), 4-1BBL (TNFSF9), FasL[1], APO2L (TNFSF10A)[15], APO3L (TNFSF12)[16], TRANCE (TNFSF11)[17], TL1 (TNFSF15)[18], or GITRL (TNFSF18)[19] (data not shown). To confirm the interaction between BLyS and TACI, we performed coimmunoprecipitation experiments (**Fig. 2**). Flag-BLyS was readily detected in anti-Fc coimmunoprecipitations with TACI-Fc, but not with Fc fusions of the human TNFR homologues HVEM (TNFRSF14)[20], DR3 (TNFRSF12)[21,22], or DR6 (TNFRSF10C)[23] (**Fig. 2a**). A substantial fraction of the added BLyS amount was coimmunoprecipitated by TACI-Fc, confirming efficient binding. Conversely, TACI-Fc was readily detected in anti-Flag coimmunoprecipitations with Flag-BLyS, whereas HVEM-Fc, DR3-Fc, or DR6-Fc were not (**Fig. 2b**). Although these results do not rule out the possibility that BLyS or TACI may bind to some other TNFR or TNF family members that were not tested, the data show that the transmembrane and soluble forms of BLyS can bind specifically to TACI.

BLyS stimulates NF-κB activity and IgM production

Many TNFR family members signal activation of the transcription factor NF-κB[2,3] Upon transfection into human embryonic kidney 293 cells, full-length human *TACI* induced NF-κB activation in a plasmid-dosage dependent manner, as determined in an NF-κB–specific reporter gene assay (data not shown). At 0.1 ng DNA, *TACI* induced only marginal activation of NF-κB (**Fig. 3**); and addition of purified human Flag-BLyS (**Fig. 3a**) or contransfection with full-length human *BLyS* (**Fig. 3b**) greatly enhanced NF-κB activation. Treatment of non transfected IM-9 cells with human Flag-BLyS also resulted in activation of NF-κB, as determined in a gel mobility shift assay (**Fig. 3c**). This activation was blocked by human TACI-Fc, but not by a control Fc fusion protein. Therefore, binding of BLyS to TACI activates NF-κB.

Previous work shows that injection of soluble BLyS into mice or expression of transgenic soluble BLyS leads to general B cell activation[4,8]. Although these studies suggest that BLyS is a positive regulator of B cell function, they fail to determine whether or not BLyS is critical for antigen-specific humoral responses. The interaction of TACI with BLyS suggested that TACI-Fc could be used to block BLyS activity *in vivo* and hence to examine the importance of this ligand for antigen-specific B cell responses.

First, we tested whether TACI-Fc could block the effect of BLyS on primary B cells *in vitro*. Human Flag-BLyS increased IgM production in cultures of human peripheral blood leukocytes (PBL) approximately

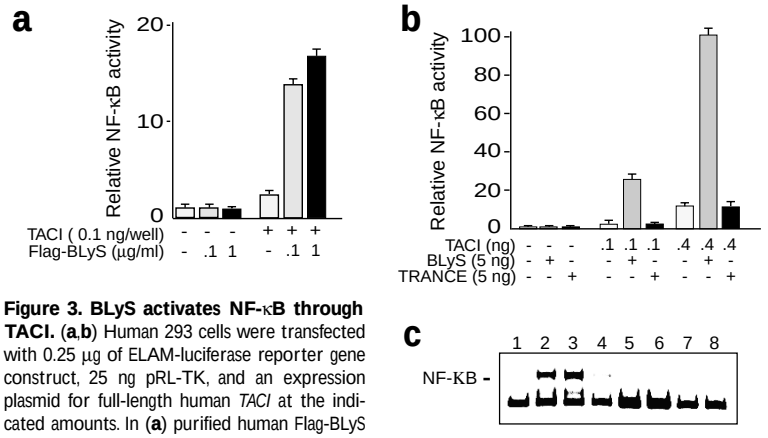

Figure 3. BLyS activates NF-κB through TACI. (**a**,**b**) Human 293 cells were transfected with 0.25 μg of ELAM-luciferase reporter gene construct, 25 ng pRL-TK, and an expression plasmid for full-length human *TACI* at the indicated amounts. In (**a**) purified human Flag-BLyS was added at the indicated concentrations 4 h after transfection; in (**b**) the cells were cotransfected with full-length human *BLyS* or *TRANCE*. Total amount of DNA was normalized with vector DNA. NF-κB activation was determined 20–24 h later by Dual-Luciferase reporter assay (Promega, Madison, WI). (**c**) Human IM-9 cells were incubated with PBS buffer (lanes 1 and 5), purified human Flag-BLyS (0.3 μg/ml) (lanes 2 and 6) or human Flag-BLyS in combination with 20 μg/ml of human TNFR1-Fc (lanes 3 and 7) or human TACI-Fc (lanes 4 and 8). NF-κB activity was measured by electrophoretic mobility shift assay using an *NF-κB*-specific DNA oligonucleotide probe (lanes 1–4), or a control, mutated oligonucleotide (lanes 5–8)[22].

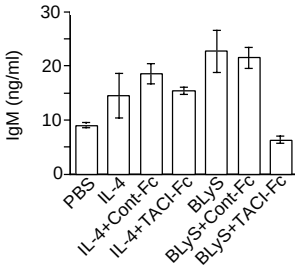

Figure 4. Inhibition of BLyS activity *in vitro* by TACI-Fc. Human donor PBL were incubated for 72 h with either: PBS buffer; IL-4 (100 ng/ml); or human Flag-BLyS (1 μg/ml) either alone or in combination with 20 μg/ml human TACI-Fc or a human IgG1 control. Cell supernatant IgM levels were analyzed by ELISA (Bethyl Laboratories, Montgomery, TX).

two-fold in a similar manner to interleukin 4 (IL-4) (**Fig. 4**). Human TACI-Fc blocked BLyS-induced IgM production completely, but it did not inhibit basal or IL-4–induced IgM formation (**Fig. 4**). Thus, TACI-Fc specifically blocks BLyS activity on primary B cells.

BLyS-TACI interaction important for antibody response

Next we tested whether blocking BLys-TACI interactions *in vivo* impairs humoral immune responses. We immunized mice with hapten-conjugated chicken γ globulin (NP_{23}-CgG), and treated them for 14 days with control murine immunoglobulin or murine TACI-Fc. TACI-Fc substantially inhibited the production of NP-specific IgM as compared to control (**Fig. 5a**), indicating that BLyS function is important during the early phase of B cell activation that leads to IgM secretion. To investigate the effect of TACI-Fc treatment on IgG1 production, we determined the levels of specific antibodies to NP_{23}-CgG and to NP_2-CgG, representing total IgG1 and the high-affinity IgG1 compartment, respectively, in the serum of immunized mice. Murine TACI-Fc inhibited the total NP-specific IgG1 response, as well as the production of high-affinity anti-NP IgG1 (**Fig. 5b,c**). Thus, BLyS activity is important not only for IgM production, but also for immunoglobulin class-switching and affinity maturation.

During the early part of an antigen-specific antibody response, B cells differentiate into antibody-forming cells (AFC). This takes place in extrafollicular areas of the spleen composed of periarteriolar lymphoid sheaths (PALS)[24,25], which is where immunoglobulin class-switching subsequently occurs. We compared the PALS-associated regions from the spleens of NP_{23}-CgG-immunized mice treated for 10 days with control murine immunoglobulin or murine TACI-Fc. As expected, control mice displayed a large number of clustered AFC foci that stained intensely with anti-IgG1 and contained many immunoblast-like cells (**Fig. 6a**, left panel). In contrast, TACI-Fc-treated mice showed only few, isolated, IgG1-positive cells, with no formation of AFC foci (**Fig. 6a**, right panel). Thus, BlyS function is important for the extrafollicular differentiation of B cells that precedes immunoglobulin class-switching in splenic PALS-associated areas.

BLyS-TACI interaction critical for GC formation

To study the potential role of BLyS-TACI interactions in antibody affinity maturation, we examined the formation of GCs in the spleens of NP_{23}-CgG–immunized mice at day 14. As expected, splenic follicles from controls displayed intense staining with peanut agglutinin (PNA), a lectin that binds specifically to GC B cells (**Fig. 6b**, left panel). In sharp contrast, splenic follicles from murine TACI-Fc–treated mice were devoid of GCs, and displayed only few, isolated, PNA-staining cells (**Fig. 6b**, right panel). Despite the lack of GCs, there were no abnormalities in the splenic follicular architecture of TACI-Fc–treated mice, as judged by staining of T cell zones with anti-CD4 antibody (green) and B cell zones with anti-B220 antibody (red) (**Fig. 6c**). This observation suggests that in TACI-Fc–treated mice some follicular B cells could differentiate into AFCs, but could not proceed to form GCs. Thus, BLyS activity is critical for proper GC formation, which may explain the impairment of antibody affinity maturation in TACI-Fc–treated mice.

Discussion

We have identified TACI as a receptor for BlyS. BLyS activation of TACI *in vitro* stimulated NF-κB and induced IgM production in B cells. Blocking BLyS activity by introducing TACI-Fc during primary immunization *in vivo* inhibited several aspects of the B cell response. These were: the early phase of extrafollicular B cell activation that leads to antigen-specific IgM production; differentiation of B cells that leads to immunoglobulin class switching; and formation of splenic GCs, where affinity maturation occurs and memory B cells are generated. Although GC formation was blocked completely by TACI-Fc, some residual IgM and IgG1 production and affinity maturation occurred. In other systems it has been observed that attenuated antibody responses can proceed despite the absence of GCs[26–28], and it is possible that other factors besides BLyS and TACI mediate the remaining antibody production. Alternatively, TACI-Fc treatment *in vivo* may not have sufficed to prevent all BLyS-TACI binding events. It is also possible that BLyS interacts with some other, yet to be identified receptor(s), which can not be blocked by TACI-Fc.

Cooperation between T and B cells is critical for GC formation; however, the precise signals and microenvironment that induce GC formation are not known. Previous studies indicate that CD40L-CD40[29] and CD86–CD28–CTLA-4[30,31] interactions are important for entry of extrafollicular B cells into GC areas and for GC establishment. Inhibition of these interactions through gene knockouts or by treatment with blocking antibodies or receptor-Fc fusion proteins diminishes antibody production and blocks GC formation[32–35]. There are some striking similarities between the BLyS-TACI and CD40L-CD40 systems. Both ligands are related to TNF and are expressed on activated T cells[4,29], and both receptors are TNFR homologues that signal through NF-κB and are expressed on B cells[5,29]. Hence, the interaction between BLyS and TACI may mediate T-cell help of B cells in a similar fashion to the interaction between CD40L and CD40[30–35]. BLyS also may contribute to the

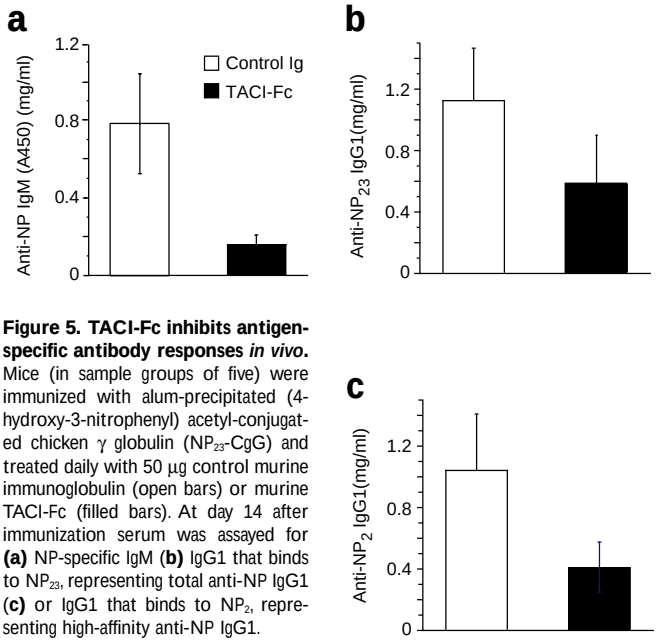

Figure 5. TACI-Fc inhibits antigen-specific antibody responses *in vivo*. Mice (in sample groups of five) were immunized with alum-precipitated (4-hydroxy-3-nitrophenyl) acetyl-conjugated chicken γ globulin (NP_{23}-CgG) and treated daily with 50 μg control murine immunoglobulin (open bars) or murine TACI-Fc (filled bars). At day 14 after immunization serum was assayed for **(a)** NP-specific IgM (**b)** IgG1 that binds to NP_{23}, representing total anti-NP IgG1 (**c)** or IgG1 that binds to NP_2, representing high-affinity anti-NP IgG1.

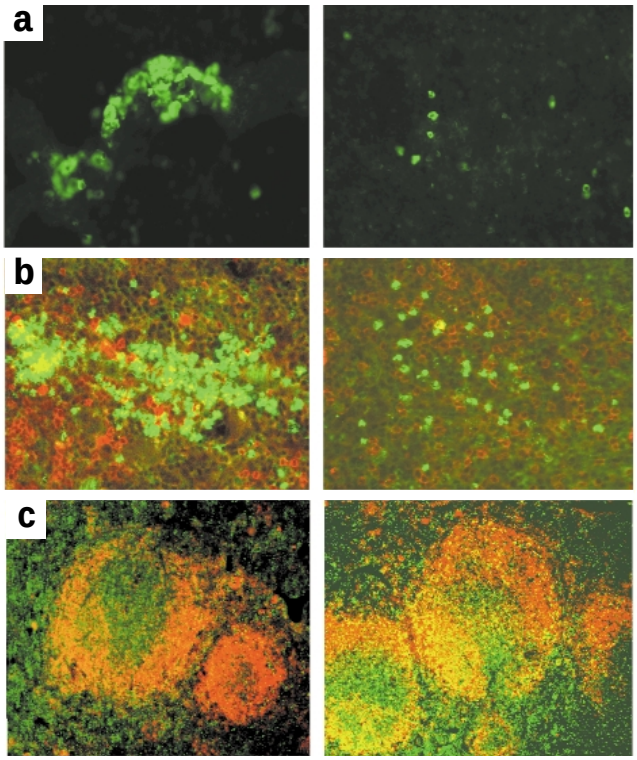

Figure 6. TACI-Fc inhibits antigen-induced splenic B cell activation and GC formation. Immunohistochemical analysis of spleens from mice treated with control murine immunoglobulin (left hand panels) or murine TACI-Fc (right hand panels) during the primary immune response. Mice were immunized and treated as in **Fig. 5. (a)** Immunoglobulin class switching in PALS-associated B cell foci: spleen sections were prepared 10 days after immunization and stained with FITC-conjugated anti-IgG1. **(b)** GC formation: spleen sections were prepared 14 days after immunization and stained with FITC-conjugated anti-PNA (green fluorescence) and Texas Red-conjugated anti-IgM (red fluorescence). **(c)** Follicular morphology: day 14 spleen sections were stained with FITC-conjugated anti-CD4 (green fluorescence) and Texas Red-conjugated anti-B220 (red fluorescence) to visualize T and B cells, respectively.

activation of B cells by dendritic cells, which do express this ligand[4]. In addition, as TACI is also expressed on activated T cells[5], BLyS may modulate directly T cell functions that support GC formation through TACI. Unlike CD40L and CD40 knockout mice, which exhibit impaired IgG but normal IgM responses, and unlike CD40L-deficient patients with hyper-IgM syndrome[36–42], TACI-Fc-treated mice showed a marked deficit in both IgM and IgG production. Thus, it is possible that BLyS and TACI operate early on in B cell activation, and blocking their action impairs all phases of the humoral response. In contrast, CD40L and CD40 may operate later in B cell activation, such that their blockade impairs only late phases of the antibody response. Alternatively, modulation of B cells by activated T cells in extrafollicular areas through CD40L-CD40 interactions may commit B cells to differentiate further to form GC in a BlyS-dependent fashion. It is possible also that BlyS-TACI interactions are important for GC maintenance and for survival of B cells in the GC, both of which are essential for an effective adaptive humoral response. Further studies will be needed to examine whether there are temporal and functional relationships between the CD40L-CD40 and the BlyS-TACI ligand receptor systems. Regardless of this, our results demonstrate that the novel molecular interaction of BLyS and TACI is critical for the antigen-specific humoral response. These observations may have implications for natural immunity, as well as for autoimmune disease.

Methods

Plasmids and transfections. Sequences encoding human BLyS (aa 136–285) and human placental alkaline phosphatase were amplified by PCR, fused and cloned into the expression vector pFlag-CMV1 (Sigma, St Louis, MO) with alkaline phosphatase at the NH_2-terminus of BLyS. AP-BLyS was expressed in human embryonic kidney 293 cells. Conditioned medium from transfected cells was filtered (0.45 μM), stored at 4 °C in a buffer containing 20 mM HEPES (pH 7.0) and 1 mM sodium azide, and subsequently used for cell staining. Flag-BLyS was constructed by cloning the same region of BLyS into pFlag-CMV1, with the Flag epitope at the N-terminus. Flag-BLyS was purified from serum-free supernatants of transfected 293 cells using anti-Flag M2-agarose (Sigma). In all experiments involving BLyS, the human version of the ligand was used.

Given that human TACI is a Type III transmembrane protein that lacks a signal peptide, we cloned the extracellular domain of *TACI* (aa 2–166) into an expression vector engineered with an NH_2-terminal signal sequence derived from pro-trypsin, and encoding the human IgG1 Fc region downstream of the *TACI* sequence as described previously for TNFR1[14]. TACI-Fc was expressed in chinese hamster ovary cells, and purified by protein A affinity chromatography. Primers based on human TACI sequence were used to amplify the mouse *TACI* cDNA from a mouse spleen library. A PCR product of about 0.45 kb was cloned. A cDNA clone containing the complete open reading frame of mouse *TACI* was subsequently isolated from the same library (Genbank accession number AF257673). Murine TACI-Fc was constructed by cloning the extracellular domain of mouse TACI (aa 2–129) between a pro-trypsin signal sequence and mouse IgG1-Fc sequence.

Expression cloning. Expression cloning was performed as described[11]. A cDNA expression library was constructed in pRK5 vector using PolyA+ mRNA from IM-9 cells. The primary transformants were divided into pools of ~1000 colonies. For each pool, a glycerol stock was prepared. Miniprep DNA (Qiagen, Valencia, CA) from each pool was transiently transfected into COS-7 cells in six-well plates using lipofectamine (GIBCO-BRL, Rockville, MD). After 36–48 h, cells were incubated with AP-BLyS conditioned medium and stained for AP activity *in situ*. A positive pool was broken down successively into smaller pools until a single positive clone was identified.

IgM production *in vitro*. Human peripheral blood mononuclear cells were isolated on a Ficol gradient (LSM media, ICN/Cappel). Peripheral blood leukocytes (PBL) were then obtained by removal of plastic-adherent cells. PBL were plated in 48-well dishes (3×10^4 cells per well in 0.3 ml of RPMI1640 medium containing 10% FBS and incubated with ligands for 72 h at 37 °C, 5% CO_2.

Antigen-specific antibody production *in vivo*. Two groups of female C57BL/6 mice aged 6 to 8-weeks-old received intraperitoneal (i.p.) immunization with 100 μg of NP_{23}-CgG (Biosource Technologies) precipitated in alum. The groups were treated daily with 50 μg of murine IgG or TACI-Fc by i.p. injection. After 14 days, mouse sera were analyzed for NP-specific IgM, or IgG1 that binds to NP_{23}-BSA, representing total anti-NP IgG1, or IgG1 that binds to NP_2-BSA, representing high-affinity anti-NP IgG1, by ELISA. Serum anti-NP IgG1 was quantified by reference to a standard curve generated with serial dilutions of an NP-specific IgG1 monoclonal antibody pEVHCγ1[30], kindly provided by Garnett Kelsoe, Duke University Medical Center. Bound antibodies were detected with AP-conjugated goat anti-mouse IgM or IgG1 (Pharmingen, San Diego). All animal experiments were performed in compliance with institutional guidelines and approved by Genentech's IACUC committee.

Immunohistochemistry. For detection of IgG1 AFCs, spleen sections were prepared 10 days after immunization, and stained with fluorescein isothiocyanate (FITC) -conjugated anti-IgG1 antibody (Pharmingen). For detection of GCs, spleen sections were prepared 14 days after immunization and stained with FITC-conjugated anti-PNA (green fluorescence) (EY Laboratories, San Mateo, CA) and Texas Red-conjugated anti-IgM (red fluorescence) (Vector Laboratories, Burlingame, CA). For detection of follicular architecture, spleen sections were stained with FITC-conjugated anti-CD4 and Texas Red-conjugated anti-B220 (Pharmingen) to visualize T and B cells, respectively.

Note added in proof: After acceptance of this manuscript, Gross *et al.* in *Nature* **404**, 995–999 (2000) reported that the TNFR homologues TACI and BCMA bind to BLyS and are implicated in models of autoimmune disease.

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