Identification of a novel receptor for B lymphocyte stimulator that is mutated in a mouse strain with severe B cell deficiency

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BLyS (also called BAFF, TALL-1, THANK, and zTNF4), a TNF superfamily member, binds two receptors, TACI and BCMA, and regulates humoral immune responses [1–7]. These two receptors also bind APRIL [7–10], another TNF superfamily member. The results from TACI$^{-/-}$ and BCMA$^{-/-}$ mice suggest the existence of additional receptor(s) for BLyS. The TACI knockout gives the paradoxical result of B cells being hyperresponsive, suggesting an inhibitory role for this receptor [11, 12], while BCMA null mice have no discernable phenotype [13]. Here we report the identification of a third BLyS receptor (BR3; BLyS receptor 3). This receptor is unique in that, in contrast to TACI and BCMA, BR3 only binds BLyS. Treatment of antigen-challenged mice with BR3-Fc inhibited antibody production, indicating an essential role for BLyS, but not APRIL, in this response. A critical role for BR3 in B cell ontogeny is underscored by our data showing that the BR3 gene had been inactivated by a discrete, approximately 4.7 kb gene insertion event that disrupted the 3′ end of the BR3 gene in A/WySnJ mice, which lack peripheral B cells.

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Results and discussion

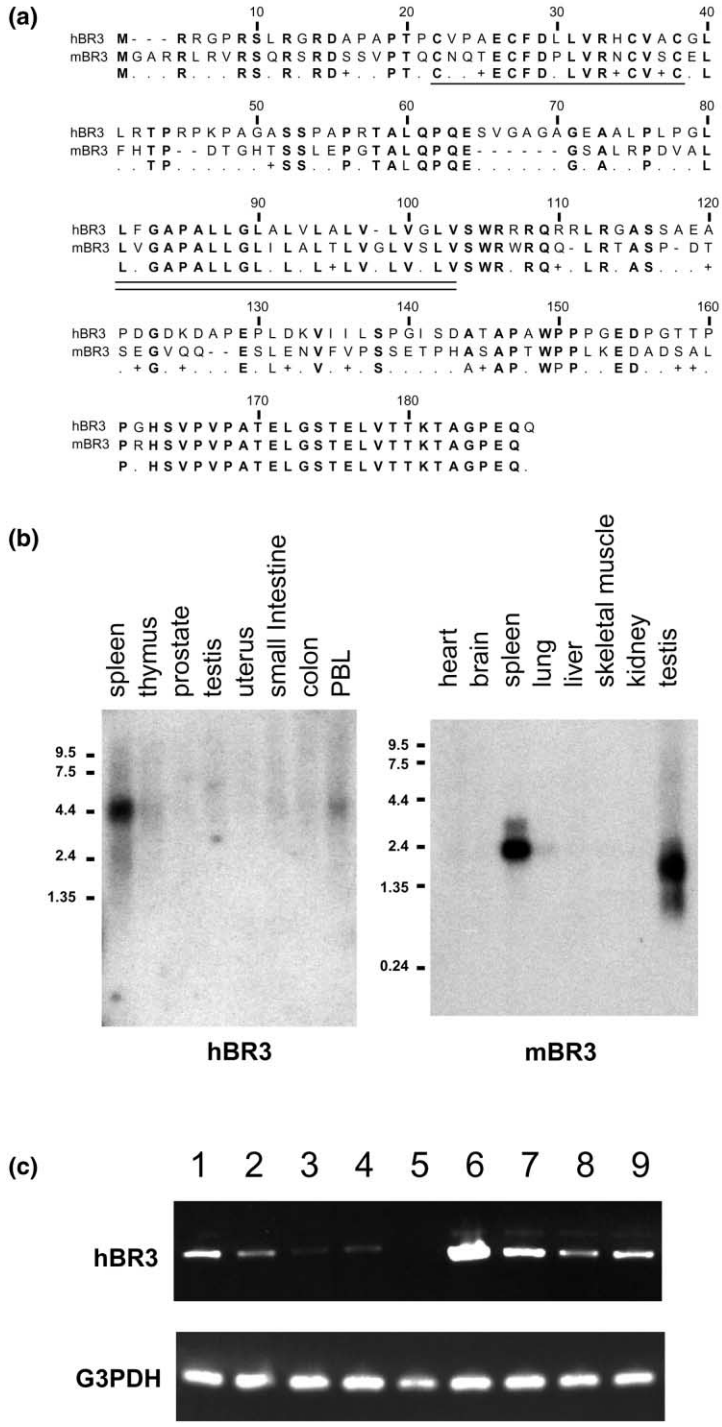
To identify additional receptor(s) for BLyS, we used an expression-cloning strategy [14, 15]. Mature soluble human BLyS (amino acids 136–285) was generated by fusion at its N terminus to human placental alkaline phosphatase (AP-BLyS). This chimeric ligand was used for screening a human spleen cDNA expression library transfected as pools into COS 7 cells [6]. As expected after the first round of screening, many positive pools contained the known BLyS receptors, TACI and BCMA, as confirmed by PCR with gene-specific primers. However, another pool was identified that contained neither TACI nor BCMA. We subjected this pool to additional rounds of screening to obtain a single cDNA clone capable of conferring BLyS binding activity to transfected COS7 cells. Sequencing revealed a single open reading frame that encoded a novel protein with a single putative transmembrane region (Figure 1a).

Database searches revealed a putative murine ortholog (GenBank accession number AK008142). Similar to human BR3 (hBR3), murine BR3 (mBR3) possesses only four cysteines. Overall, hBR3 and mBR3 exhibited 56% identity and 66% similarity. Both hBR3 and mBR3 lack an N-terminal putative signal peptide, indicating that they are type III transmembrane proteins [16]. The intracellular domain of BR3 is highly conserved between hBR3 and mBR3 (Figure 1a). Northern blot analysis indicated that both hBR3 and mBR3 were highly expressed in the spleen (Figure 1b).

The prominent expression of BR3 in the spleen and in particular by B cells is consistent with it being a B cell receptor for BLyS. PCR analysis of a human cDNA panel showed highest expression of hBR3 in resting CD19+ B cells (Figure 1c), consistent with BR3 being a receptor for BLyS on B cells. However, the expression pattern of BR3 is distinct from that of TACI and BCMA. While BCMA is B cell specific and TACI is expressed both by B cells and by activated T cells [17–20], BR3 is highly expressed by resting B cells and is also detectable in resting CD4+ T cells. Additionally, in contrast to TACI, BR3 appears to be downregulated upon activation (Figure 1c).

Transfection of hBR3 or mBR3 expression constructs conferred strong binding to AP-BLyS, but not AP-APRIL (Figure 2a and data not shown). In contrast, both AP-BLyS and AP-APRIL bound TACI-transfected cells (Figure 2a, lower panel). A human Fc fusion protein based on the hBR3 ectodomain (hBR3-hFc) bound cells transfected with a plasmid encoding the full-length transmembrane form of BLyS, but not cells expressing APRIL (Figure 2b). Murine BR3-Fc, like hBR3-hFc, also only bound BLyS-transfected but not APRIL-transfected COS 7 cells (data not shown). To confirm the interaction between BR3 and BLyS, we performed coimmunoprecipitation (co-IP)

Figure 1

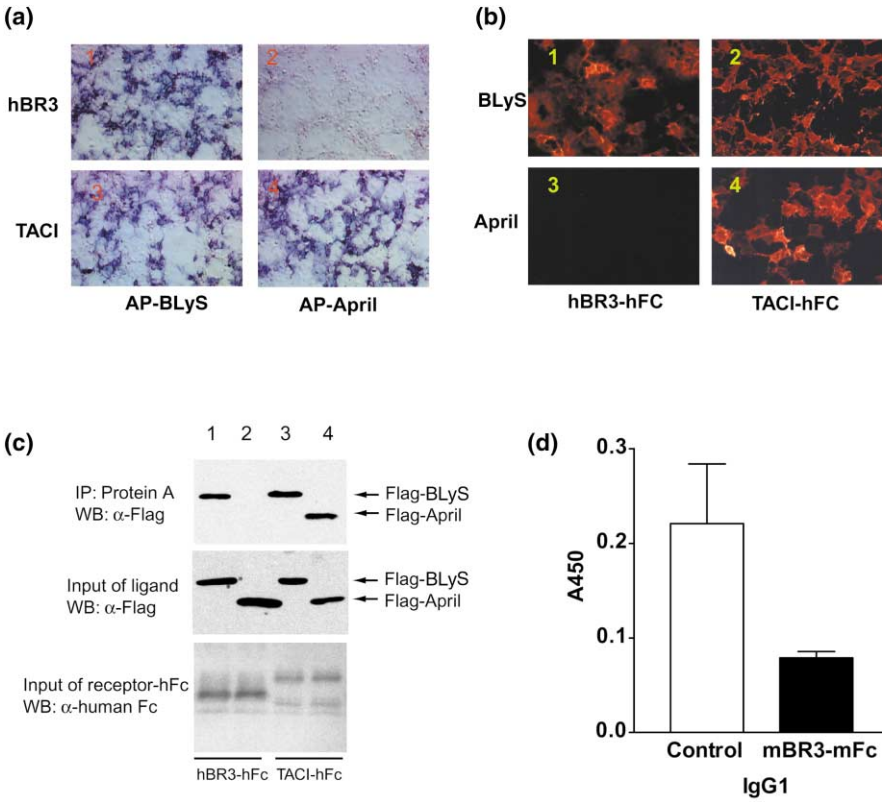


(a) Sequence alignment of human and murine BR3. Amino acids that are identical in human and murine BR3 are shown in bold. Conserved amino acids are indicated by a plus sign. The region containing four cysteine residues is underlined, and the predicted membrane-spanning region is doubly underlined. **(b)** Northern blot analysis of BR3. Human (left) and mouse (right) multiple tissue Northern blots (Clontech) were probed with ^{32}P-labeled cDNA fragments corresponding to the coding region of human or murine BR3. **(c)** PCR analysis of human multiple tissue cDNA panel (Clontech). cDNA fragments were amplified with gene-specific primers. Lanes 1–9: 1, PBL; 2, resting CD4+ cells; 3, activated CD4+ cells; 4, resting CD8+ cells; 5, activated CD8+ cells; 6, resting CD19+ cells; 7, activated CD19+ cells; 8, lymph node; 9, spleen.

experiments (Figure 2c). Flag-BLyS but not Flag-APRIL was readily detected in complex with hBR3-hFc, whereas TACI-hFc bound both Flag-BLyS and Flag-APRIL. Furthermore, BR3-Fc failed to bind cells expressing several other TNF family members, including CD27L, CD30L, CD40L, EDA-A1, EDA-A2, 4-1BBL, FasL, Apo2L/TRAIL, Apo3L/TWEAK, OX-40L, RANKL/TRANCE, and GITRL (data not shown). These results show that, unlike TACI and BCMA, BR3 specifically binds BLyS but not APRIL.

It has previously been shown that treating antigen-challenged mice with TACI-Fc or BCMA-Fc suppresses antibody production [6]. Since TACI-Fc and BCMA-Fc bind

Figure 2



BR3 is a specific receptor for BLyS but not for APRIL. **(a)** COS 7 cells transfected with hBR3 (1, 2) or TACI (3, 4) were incubated with conditioned medium containing AP-BLyS (1, 3) or AP-APRIL (2, 4). Cells were washed, fixed, and stained for the AP activity in situ. **(b)** COS 7 cells transfected with BLyS (1, 2) or APRIL (3, 4) were incubated with BR3-hFc (1, 3) or TACI-hFc (2, 4). Cells were washed and fixed, and the bound receptor-hFc protein was detected with a biotinylated goat anti-human antibody followed by Cy3-streptavidin. **(c)** BR3-hFc (lanes 1, 2) or TACI-hFc (lanes 3, 4) were incubated with Flag-BLyS (lanes 1, 3) or Flag-APRIL (lanes 2, 4). The receptor-Fc fusion proteins precipitated with protein-A-agarose were subjected to immunoblotting with anti-Flag antibody. Equivalent amounts of ligand (middle panel) or receptor-hFc (bottom) were used in the binding experiment. **(d)** TNP-specific IgG1 production from control Ig-treated (white bar) and from BR3-Fc-treated (black bar) mice. Data represent five mice in each group, given in mean $\pm$ SEM, $p < 0.005$.

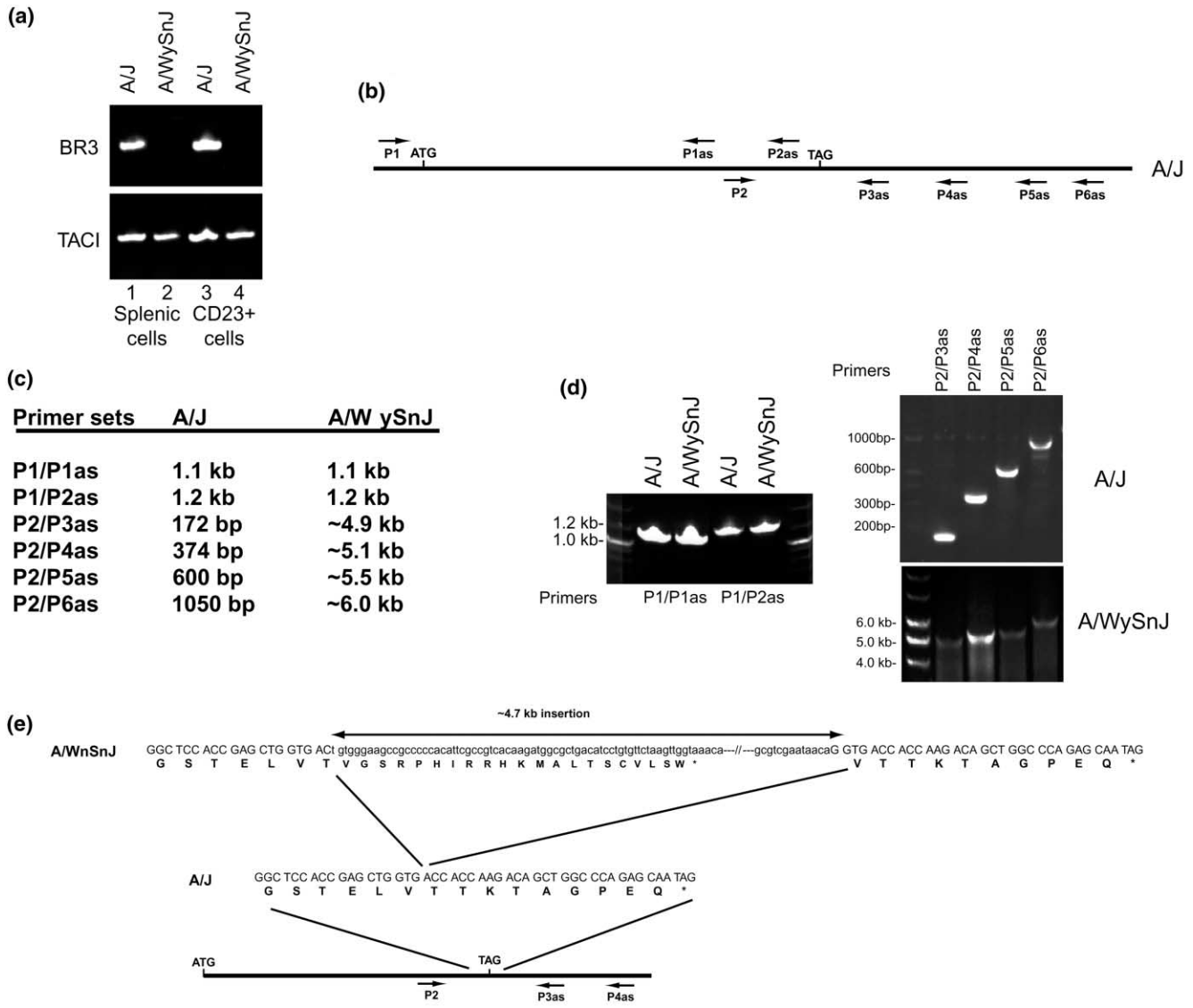
both BLyS and APRIL, it was only possible to surmise that either BLyS or APRIL or both were important in mediating the antibody response. However, with the availability of BLyS-specific BR3-Fc, it is possible to distinguish the in vivo roles of BLyS and APRIL in the B cell response to antigen challenge. BR3-Fc, in contrast to control-Fc, substantially inhibited the production of TNP-specific IgG1 in mice immunized with TNP-Ficoll (Figure 2d).

The human BR3 gene maps to chromosome 22, which is syntenic to the middle region of chromosome 15 in mice (data not shown). Interestingly, a gene locus termed Bcmd (B cell maturation defect) also has been reported to map to the middle region of mouse chromosome 15, which is responsible for the profound defect in the B cell maturation in the mouse A/WySnJ strain. A related A/J strain, however, which does not possess a single autosomal codominant Bcmd locus, has no deficit in peripheral B cells [21–23]. To determine if the gene defect in A/WySnJ mice, as defined genetically by the Bcmd locus, was in the gene encoding BR3, we performed RT-PCR to detect the transcript for BR3. RT-PCR analysis failed to reveal the presence of BR3 transcript corresponding to the complete coding region in splenic or CD19+ B cell RNA from A/WySnJ mice, while the transcript for TACI control was easily discernable (Figure 3a). This result suggested alteration of the BR3 gene in A/WySnJ mice.

To determine the nature of the BR3 gene mutation, we designed a PCR strategy based on the genomic sequence to amplify products of various lengths, as outlined in Figure 3b. When primers P1 (18 bp upstream of ATG) and P1AS or P2AS (36 bp upstream of TAG) were used for amplification, PCR products from the A/J and A/WySnJ strains were the same size (Figure 3c,d). This result indicates that the BR3 gene up to 36 bp upstream of the TAG (stop codon) is unaltered in the A/WySnJ strain. However, when the region from 109 bp upstream of TAG to 14 bp downstream of TAG was amplified with primers P2 and P3AS, a PCR product of expected size (172 bp) was amplified from the A/J strain, but the A/WySnJ strain yielded an approximately 4.9 kb product. This result indicates that there was an insertion of approximately 4.7 kb in the region between P2AS and P3AS, which are 53 bp apart. Additional primer sets P2/P4AS, P2/P5AS, and P2/P6AS all amplified products of expected size, as shown in Figure 3c, confirming insertion of an approximately 4.7 kb in the BR3 gene of A/WySnJ mice.

To determine the exact location of the insertion within the BR3 gene of A/WySnJ mice, we amplified and subse-

Figure 3



Analysis of BR3 transcripts and gene in A/WySnJ mice. **(a)** Analysis of BR3 transcripts in splenic cells (lanes 1, 2) or CD23+ B cells (lanes 3, 4). RT-PCR was performed with total RNA isolated from A/J (lanes 1, 3) or A/WySnJ mice by the use of (top) murine BR3- or (bottom) murine TACI-specific primers. **(b)** Location of primers used in PCR analysis of the BR3 gene. The initiation and stop codons are indicated as ATG and TAG, respectively. **(c,d)** Primers as indicated were used for amplifying genomic DNA isolated from A/J or A/WySnJ mice. The sizes of the PCR products are summarized in (c). **(e)** Insertion mutation of BR3 gene in A/WySnJ mice. Sequencing of the PCR (Primers P2 and P4AS) product amplified from genomic DNA of A/WySnJ mice revealed an insertion event that deleted the last eight amino acids in the C-terminal coding region of the BR3 gene.

quently sequenced a 5.1 kb PCR product from A/WySnJ mice with primers P2 and P4AS. Alignment of the A/J and A/WySnJ DNA sequences revealed that the BR3 gene in A/WySnJ mice is missing a region encoding the last eight amino acids of the gene (Figure 3e).

Recent reports have established a key role for BLyS in the regulation of humoral immunity; however, the individual contributions of its receptors, TACI and BCMA, are still unknown. Rather than defining the discrete roles of these receptors, results from TACI and BCMA knockout mice have added additional complexity to the system. The TACI null mutation leads to massive accumulation and activation of B cells in vivo, while $BCMA^{-/-}$ mice have no obvious phenotype [11–13]. Thus, $TACI^{-/-}$ mice give the paradoxical result of hyperproliferation, suggesting an inhibitory role for this receptor, while the significance of BCMA remains to be defined. These results clearly indicate the existence of an additional receptor(s) for BLyS. In the present report, a novel BLyS receptor identi-

fied by expression cloning is described that plays a key role in B cell development and function.

Although BR3, like TACI and BCMA, binds BLyS and is expressed on B cells, this receptor is unique in other features. First, sequence alignments indicate that BR3 is not a typical member of the TNF receptor superfamily, which is defined by the presence of characteristic cysteine-rich repeats within the extracellular ligand binding domain. These pseudorepeats (40 amino acids) are typically defined by three intramolecular disulfide bridges formed by six highly conserved cysteines [24]. BR3 has only four cysteine residues in its ectodomain. It should be noted, however, that TACI, BCMA, and FN14 also contain a cysteine-rich module composed of four cysteine residues with the consensus sequence $xxCx_{2-3}Cx_{5-8}CxxCxx$. BR3 contains a similar pattern of cysteines except for the first cysteine, which is followed by four amino acids. This suggests that the overall tertiary structure of these receptors may be similar.

Additional unique features of BR3 are that it only binds BLyS and that treatment of antigen-challenged mice with BR3-Fc inhibits antibody production, indicating an essential role for BLyS, but not APRIL, in this response. These results underscore the importance of BLyS in the B cell response and suggest no major role for APRIL in antigen-specific antibody production. Furthermore, the data also support the importance of BR3 in B cell-mediated effects.

Compelling data for the functional significance of BR3 come from the study of B cell-deficient A/WySnJ mice that, unlike the related A/J strain, possess a single autosomal codominant locus, termed Bcmd, that is responsible for the profound deficit in peripheral B cells [21–23]. Splenic B cells from A/WySnJ mice do not exhibit a proliferative response to recombinant BLyS either in vitro or in vivo (our submitted manuscript). Additionally, A/WySnJ mice display normal expression of the known BLyS receptors, TACI and BCMA. Given this, it was hypothesized that the gene defect in A/WySnJ mice, as defined genetically by the Bcmd locus, was in the gene encoding BR3. RT-PCR analysis failed to reveal the presence of a BR3 transcript corresponding the complete coding region in splenic or B cell RNA from A/WySnJ mice, while the transcript for TACI control was easily detectable in the same samples. It is further shown that the BR3 gene has been inactivated by a discrete, approximately 4.7 kb gene insertion event that disrupts the 3′ end of the gene in A/WySnJ mice. Defining the boundaries of the insertion allowed the conclusion that the only gene disruption in A/WySnJ mice was BR3.

In sum, these data are consistent with an inactivation of BR3 being responsible for the lack of peripheral B cells observed in A/WySnJ mice. The signaling pathway engaged by BR3 is likely responsible for the B cell-proliferative effect of BLyS, and in its absence, B cell homeostasis is compromised.

Materials and methods

cDNA expression vectors

A sequence encoding human placental alkaline phosphatase (AP) and either human BLyS (amino acids 136–285) or human APRIL (amino acids 105–250) was amplified by PCR and cloned in frame into the expression vector pFlag-CMV1 (Sigma) with AP at the N terminus of BLyS or APRIL (AP-BLyS/AP-APRIL). Expression constructs for Flag-BLyS or Flag-APRIL were generated by direct cloning of BLyS (amino acids 136–285) or APRIL (amino acids 105–250) into pFlag-CMV1. hBR3-hFc and TACI-hFc were constructed by the cloning of hBR3 (amino acids 2–62) or TACI (amino acids 2–166) into the pRK5 vector engineered with an upstream heterologous signal sequence (pre-protrypsin amino acids 1–17 from pFlag-CMV1) and a downstream human IgG1 Fc domain.

Expression cloning of BR3

Expression cloning was performed as described [6]. 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. A cDNA expression library was constructed with PolyA+ mRNA from human spleen. The primary transformants were divided into pools of approximately 1000 colonies. For each pool, a glycerol stock was prepared. Miniprep DNA (Qiagen) from each pool was transiently transfected into COS 7 cells in 12-well plates by the use of lipofectamine (GIBCO-BRL). After 36-48 hr, 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.

In vivo antigen-specific antibody production

Two groups (five per group) of 5-week-old C57BL/6 mice were immunized intraperitoneally (i.p.) with 100 μg of TNP-Ficoll (Biosource Technologies). One group was given 100 μg of control Ig, while the other group received 100 μg of BR3-Fc daily. Seven days later, total TNP-specific IgG1 present in the mice sera was measured. TNP-specific IgG1 antibodies were quantified with ELISA plates coated with TNP-conjugated BSA (Biosource Technologies). Bound antibodies were detected with alkaline phosphatase-conjugated goat anti-mouse IgG1 (Pharmingen).

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