Identification of proteoglycans as the APRIL-specific binding partners

Karine Ingold,1 Adrian Zumsteg,1 Aubry Tardivel,1 Bertrand Huard,2 Quynh-Giao Steiner,1 Teresa G. Cachero,3 Fang Qiang,2 Leonid Gorelik,3 Susan L. Kalled,3 Hans Acha-Orbea,1 Paul D. Rennert,3 Jürg Tschopp,1 and Pascal Schneider1

1Department of Biochemistry, University of Lausanne, CH-1066 Epalinges, Switzerland
2Department of Dermatology, University of Geneva Medical Center, CH-1211 Geneva 4, Switzerland
3Biogen Idec, Cambridge, MA 02142

B cell activating factor of the tumor necrosis factor (TNF) family (BAFF) and a proliferation-inducing ligand (APRIL) are closely related ligands within the TNF superfamily that play important roles in B lymphocyte biology. Both ligands share two receptors—transmembrane activator and calcium signal–modulating cyclophilin ligand interactor (TACI) and B cell maturation antigen (BCMA)—that are predominantly expressed on B cells. In addition, BAFF specifically binds BAFF receptor, whereas the nature of a postulated APRIL-specific receptor remains elusive. We show that the TNF homology domain of APRIL binds BCMA and TACI, whereas a basic amino acid sequence (QKQKKQ) close to the NH2 terminus of the mature protein is required for binding to the APRIL–specific “receptor.” This interactor was identified as negatively charged sulfated glycosaminoglycan side chains of proteoglycans. Although T cell lines bound little APRIL, the ectopic expression of glycosaminoglycan-rich syndecans or glypicans conferred on these cells a high binding capacity that was completely dependent on APRIL’s basic sequence. Moreover, syndecan–1–positive plasma cells and proteoglycan–rich nonhematopoietic cells displayed high specific, heparin-sensitive binding to APRIL. Inhibition of BAFF and APRIL, but not BAFF alone, prevented the survival and/or the migration of newly formed plasma cells to the bone marrow. In addition, costimulation of B cell proliferation by APRIL was only effective upon APRIL oligomerization. Therefore, we propose a model whereby APRIL binding to the extracellular matrix or to proteoglycan–positive cells induces APRIL oligomerization, which is the prerequisite for the triggering of TACI– and/or BCMA–mediated activation, migration, or survival signals.
mote proliferation of certain cell lines, including fibroblasts and malignant glioblastoma (8, 9). Administration of BCMA:Fc retards tumor growth in nude mice injected with human colon carcinoma HT29 cells and, to a lesser extent, human lung carcinoma A549 cells (3). Recombinant APRIL binds to several cell lines that do not express detectable mRNA for TACI and BCMA, suggesting that an additional APRIL-specific receptor exists. The binding to this receptor is reduced by predepletion of APRIL with BCMA:Fc, but it is not competitively inhibited when APRIL and BCMA:Fc are added to cells simultaneously. This suggests that the APRIL-specific receptor expressed on nonhematopoietic cells binds APRIL with a much higher affinity than BCMA, or at a distinct binding site (3). Both APRIL and BAFF are released in a soluble form by proteolytic processing at a furin consensus sequence (R X R/K R; reference 1). This leaves a short NH₂-terminal extension in front of the TNF homology domain in the cleaved, mature forms of APRIL and BAFF. In the case of APRIL, this sequence is basic in nature (Fig. 1 A). We show that the basic NH₂-terminal sequence of APRIL allows binding to sulfated glycosaminoglycans, which most likely represents the proposed, but as yet uncharacterized, APRIL-specific binding partner.

RESULTS
The mature NH₂-terminal sequence of APRIL is required for binding to the APRIL-specific binding partner
We have previously shown that APRIL displayed significant binding to various cell lines that did not express the two known APRIL receptors, TACI and BCMA. Consistent with the absence of BCMA and TACI, these cells did not interact with BAFF, suggesting the existence of an additional, widely distributed receptor for APRIL with little or no affinity for BAFF (3). The mature form of APRIL (designated here as APRIL A88) starts at Ala 88 and contains 10 amino acids in front of the TNF homology domain (Fig. 1 A). The removal of this sequence was initially performed to assess the impact on the production yield of APRIL, which was indeed increased about threefold (unpublished data). APRIL H98 retained its ability to bind both BCMA and TACI, but this deletion completely abolished binding to the putative endogenous APRIL-specific binding partner on the same cells (Fig. 1 B, ligand only). Importantly, these results were also obtained with the murine proteins (unpublished data).

The staining pattern of BAFF and APRIL was examined in the presence of soluble BCMA, which acted as a decoy receptor and prevented the binding of APRIL H98 and APRIL A88 to cell-associated TACI and BCMA. Soluble BCMA also abolished the binding of BAFF to cell-associated BCMA and reduced the binding to BAFF-R and TACI. This was consistent with the fact that BAFF has a lower affinity for BCMA than for BAFF-R and TACI. However, soluble BCMA did not affect the binding of APRIL A88 to the endogenous APRIL-specific interactors (Fig. 1 B, ligand only). A control decoy receptor, soluble CD40, had no effect (unpublished data). These studies indicate that the binding of APRIL A88 to the APRIL-specific binding partner requires a sequence within the 10 NH₂-terminal amino acids of mature APRIL, which is distinct from the binding site to BCMA and TACI.

Heparin competes with the APRIL-specific interactor for the binding of APRIL
The mature NH₂-terminal sequence of APRIL contains a cluster of basic amino acid residues that are absent in BAFF...
(Fig. 1 A). To test whether this cationic stretch of amino acids could interact with negatively charged structures, such as phospholipids or anionic sugars, on the cell surface, the highly negatively charged heparin polymer was added during the staining procedure. Heparin did not affect the binding of BAFF and APRIL to BCMA and TACI, but specifically abolished the binding of APRIL A88 to the endogenous APRIL-specific interactor (Fig. 1 B, ligand + heparin). As expected from this result, the combination of soluble BCMA and heparin strongly reduced the binding of APRIL A88 to the endogenous APRIL-specific interactor, as well as BCMA and TACI (Fig. 1 B).

The competition with heparin suggested that APRIL A88 might bind heparin directly. Interaction studies confirmed that Flag-tagged APRIL and BAFF constructs bearing various deletions at the mature NH$_2$ terminus all bound BCMA:Fc, but the binding to heparin-Sepharose required the basic sequence that was mapped down to six amino acids (sequence 92–97, QKQKKQ). Importantly, APRIL processed by endogenous furin also interacted with heparin, ruling out the possibility that the binding was contributed by the Fc or Flag tags in other recombinant constructs (Fig. 2). A chimeric ligand with the mature NH$_2$-terminal sequence of APRIL fused to BAFF failed to bind heparin and the endogenous APRIL-specific interactor (Fig. 2 and not depicted). In addition, a synthetic peptide comprising amino acids 88–99 of APRIL did not competitively inhibit the binding of APRIL A88 to the endogenous APRIL-specific interactor (unpublished data). This suggests that the basic NH$_2$-terminal sequence of APRIL is necessary, but not solely sufficient, for binding to heparin and to the endogenous APRIL-specific binding partner. Based on the crystal structure of the APRIL–BCMA complex (10), three basic amino acid residues of APRIL were substituted by those found at the corresponding positions of BAFF (R129S, R172S, and H203E). These residues contribute to a basic surface on APRIL that is distinct from the binding site for BCMA and TACI (Fig. 2 C). Their mutation specifically affected binding to heparin, but not to BCMA (Fig. 2 A), strongly suggesting that both the basic surface and the basic NH$_2$-terminal sequence of APRIL are required for heparin binding. Interestingly, the mature NH$_2$-terminal sequences of BAFF and APRIL significantly affected SDS-PAGE migration, with the BAFF sequence resulting in a higher apparent molecular mass than the APRIL sequence (Fig. 2). This may reflect differences in SDS binding or indicate a rigid conformation of the BAFF sequence. Curiously, the BAFF sequence GPEET is found as a repetitive sequence in procyclin, which is a surface antigen of a trypanosomatid protozoan parasite known to migrate with an abnormally high apparent molecular mass by SDS-PAGE (11).

**Figure 2.** The NH$_2$ terminus of mature APRIL and other basic residues are required for heparin binding. (A) Flag-tagged ligands were immunoprecipitated with either BCMA:Fc or heparin-Sepharose. Naturally processed, untagged APRIL was also used. Proteins were detected by immunoblot with anti-Flag or anti-APRIL mAbs as indicated. (B) Schematic representation of the constructs used in A. (C) Structure of the APRIL–BCMA complex (reference 10), showing one subunit of the trimer. Side chains of all basic amino acid residues that are present on the upper surface of APRIL are shown (gray, common to APRIL and BAFF; black, APRIL specific [R129, R172, and H203]). The basic NH$_2$ terminus of mature APRIL, which is only partially apparent in the crystal structure, comprises K97. A heparin fragment is shown above its proposed binding site. APRIL, BCMA, and heparin were drawn using the PDB atomic coordinate files 1XU2 and 1FQ9.

Sulfated glycosaminoglycan side chains of proteoglycans are the APRIL-specific binding partner

Heparin consists of alternating residues of glucuronic acid (or its 5-epimer iduronic acid) and N-acetyl-galactosamine in which both sugar residues can be mono- or disulfated, thereby providing additional negative charges. Because of its soluble nature, heparin alone is not likely to be the endogenous APRIL-specific binding partner, but heparin-like glycosaminoglycans are present in membrane-bound proteoglycans. Treatment of 293T cells with a sulfation inhibitor, chlorate, resulted in markedly decreased binding of both mouse and human APRIL A88, but did not affect the binding of APRIL to transfected BCMA (Fig. 3 A and not depicted). To demonstrate more directly the interaction of APRIL A88 with proteoglycans, Jurkat cells were selected because they express relatively low levels of the endogenous APRIL-specific interactor. Transfected Jurkat cells expressing various proteoglycans (syndecan-1, -2, or -4, or the gly-
colipid-anchored glypican-1) gained robust and specific binding to APRIL A88 (Fig. 3 B). This indicates that the binding is dependent on the glycosaminoglycan side chains rather than on specific interactions with the polypeptide portion of either syndecans or glypicans. Collectively, our results strongly suggest that the endogenous APRIL-specific binding partners expressed by 293T and other cell lines are sulfated glycosaminoglycan side chains of cell surface proteoglycans.

Expression of APRIL and BAFF receptors in cell lines and mouse lymphocytic cells

We determined the binding patterns of BAFF and APRIL on various cell lines, taking advantage of the possibility to efficiently inhibit APRIL binding to glycosaminoglycans with heparin. The high binding of APRIL to nonhematopoietic cell lines, such as HT-29 and SW480 colorectal adenocarcinomas, A549 lung carcinoma, 293 embryonic kidney cells, and NIH-3T3 murine fibroblasts, was completely inhibited by heparin. BAFF did not bind these cell lines, indicating a proteoglycan-dependent binding (Fig. 1 and not depicted). In Burkitt lymphoma BJAB cells, the moderate binding of APRIL was proteoglycan-dependent, whereas BAFF gave a strong specific binding, consistent with the high expression of BAFF-R in these cells (12). The APRIL staining of the TACI-positive IM-9 B lymphoblast cells (13) was caused by both heparin-sensitive and heparin-insensitive (TACI) sites.

Figure 3. APRIL binds the sulfated glycosaminoglycan side chains of proteoglycans. (A) 293T cells grown in the presence or absence of the sulfation inhibitor chlorate were stained with Fc versions of the indicated human (h) or murine (m) APRILs. (B) Jurkat cells cotransfected with various human syndecan or glypican expression constructs and an EGFP tracer were stained with Fc versions of human BAFF, APRIL H98, or APRIL A88. Both axes show fluorescent intensity on a logarithmic scale (10^1–10^4).

Figure 4. APRIL binding to primary syndecan-1–positive plasma cells. (A) Flow cytometry analysis of draining popliteal lymph nodes from mouse mammary tumor virus–infected mouse. B220 and CD138 stainings were used to define B cell (B), plasma cell (PC), and T cell and other cell (T) populations as indicated. (B) Staining of cell populations defined in A with Fc versions of mouse BAFF or APRIL A88, in the presence or absence of heparin.

We investigated the binding of BAFF and APRIL to primary lymphocytes from lymph nodes in which large numbers of plasma cells had been elicited by infection with a mouse mammary tumor virus (15). BAFF bound lymph node B cells and plasma cells, but not T cells. APRIL bound B, T, and plasma cells. In the presence of heparin, binding to T and B cells was abolished, but specific APRIL binding on plasma cells was maintained. This suggests that B cells express mainly BAFF-R, whereas plasma cells express BAFF-R, TACI, and/or BCMA (including at least one of the latter two receptors), in addition to proteoglycans (Fig. 4). Together, these results indicate that APRIL binding to nonhematopoietic cells is glycosaminoglycan mediated. The same holds true for hematopoietic cells, except that binding to TACI and/or BCMA in plasma cells and TACI-positive cell lines also contributes to the binding.

APRIL oligomerization is required for B cell costimulation

Although BAFF and APRIL costimulate the proliferation of splenic B cells (16), we initially observed this effect with BAFF but not APRIL. We therefore wondered whether APRIL, like CD40L, might require a higher order oligomer-
ization in order to successfully deliver a coproliferative signal (17). Indeed, antibody-mediated cross-linking of APRIL and CD40L induced a significant costimulation of B lymphocyte proliferation, whereas BAFF, though potentiated by cross-linking, was already active on its own. A control ligand, ectodysplasin A (EDA), did not costimulate B cells under any conditions (Fig. 5 A). We also tested for the involvement of the glycosaminoglycan binding site of APRIL by adding heparin in the assay, based on the hypothesis that heparin could cross-link APRIL and render it signaling competent. However, only minor costimulation of B cell proliferation was obtained with APRIL A88 plus heparin, suggesting that heparin is not an optimal cross-linker under our experimental conditions (Fig. 5 A). Glycosaminoglycan-dependent oligomerization was not observed at all with APRIL H98 and CD40L (Fig. 5). Heparin did not further enhance the activity of APRIL cross-linked with antibodies (unpublished data).

The costimulatory effect of cross-linked APRIL H98 cannot be mediated by BAFF-R or proteoglycans, which are not recognized by this ligand. Transitional type 2 B cells and marginal zone B cells, which are specific to the spleen, express TACI constitutively and may therefore represent a target for APRIL (18, 19). However, the observation that APRIL costimulated blood and lymph node B cells, in addition to splenic B cells (Fig. 5 B), suggests that inducible TACI (18) may mediate APRIL costimulatory effects.

Impaired generation of bone marrow plasma cells upon blockade of APRIL and BAFF but not BAFF alone

We investigated the possible implications of APRIL in the biology of newly formed plasma cells. For this purpose, an antibody response was first elicited with nitrophenyl-conjugated chicken gammaglobulin (NP-CGG) in alum. This model antigen induces a germinal center reaction after 5–7 d (20), which is followed by the migration of plasma cells to the bone marrow where they can be detected as early as day 11 after immunization. Mice were treated 6 d after immunization with either BCMA:Fc (which blocks both BAFF and APRIL) or BAFF-R:Fc (which blocks only BAFF) and analyzed 5 d later. This regimen blocked the treatment’s impact on antigen-specific precursor cells and specifically assessed the role of BAFF and APRIL depletion in the fate of newly formed plasma cells. Although the treatment reduced the total number of splenic IgG1-switched plasma cells and the IgG1 antibody titer in serum about twofold, we noticed no differences between BAFF-R:Fc and BCMA:Fc treatments, suggesting that this effect was BAFF mediated (Fig. 6, A and C). However, the frequency of specific IgG1-secreting plasma cells was significantly reduced in mice treated with BCMA:Fc (Fig. 6, B and C). This result indicates that APRIL is important for the migration or survival of newly formed plasma cells to the bone marrow.
plasma cells in the bone marrow was dramatically decreased in mice treated with BCMA:Fc, but not BAFF–R:Fc. This suggests that APRIL, either alone or together with BAFF (but not BAFF alone), is required for the migration and/or maintenance of bone marrow resident cells early in the primary immune response (Fig. 6 B).

DISCUSSION

After the identification of APRIL and BAFF some years ago, several laboratories have attempted to identify their cognate receptors. Interestingly, published results using unbiased expression cloning approaches have invariably been performed with BAFF and not APRIL (12, 16, 21–26). Expression cloning is strongly facilitated by a low background of the screening ligand, a condition that is not fulfilled by APRIL which binds to most cells via glycosaminoglycan interactions. Although the binding of proteins to heparin or to heparansulfate is a frequent event, it has not been previously described in the TNF family. Is it therefore legitimate to wonder whether APRIL is unique in this respect, or whether glycosaminoglycan binding is an important intrinsic feature within the TNF family. In contrast to APRIL, BAFF does not interact with glycosaminoglycans, but at least one other TNF family member, EDA, does (unpublished data). It remains to be determined whether other ligands share this property.

We have mapped the heparin-binding region within the NH₂-terminal sequence of mature APRIL. However, this region alone is insufficient to mediate glycosaminoglycan binding, which suggests that additional cationic features of APRIL are involved. Indeed, the surface of APRIL that harbors the basic mature NH₂-terminus also exposes several additional basic amino acids (Arg129, Arg172, and His203; reference 10) that are required for efficient binding to heparin, and that are absent at the corresponding positions of BAFF. These data strongly suggest the existence of an extended glycosaminoglycan binding site in APRIL (Fig. 2 C). It is frequently observed that heparin-binding sites do not only rely on linear amino acid sequences but also on patches of amino acid residues scattered over the protein surface, as is the case with many chemokines (27). Although APRIL contains another basic surface at the site contacted by BCMA (28), it is unlikely to participate in glycosaminoglycan recognition because it lies on the opposite face of APRIL and competition with BCMA was not observed.

TNF family ligands adopt a homotrimeric structure that is competent for receptor binding. However, binding to receptors may not be sufficient to induce productive signaling within the cell. Indeed, a higher order oligomerization of several trimeric TNF family ligands, such as FasL and CD40L, is required for the efficient induction of a biological response (17, 29). It is believed that the cross-linking of soluble trimeric ligands mimics the membrane-bound form of the ligand. Our results indicate that APRIL belongs to the category of TNF ligands that requires cross-linking to exert activity, at least with respect to B cell costimulation. This is, however, difficult to reconcile with the observation that APRIL is entirely released in a putatively inactive soluble form after intracellular processing (30). It is therefore tempting to propose that soluble APRIL, cross-linked to cell-associated or matrix proteoglycans by virtue of its heparansulfate-binding site, may regain an activity similar to that of the membrane-bound form. Heparansulfates can provide or reinforce physical links between proteins. For instance, heparin as an anticoagulant agent not only induces conformational changes in antithrombin III, resulting in the exposure of the reactive site loop that acts as a bait for active thrombin, but also bridges thrombin with its inhibitor (31, 32). Similarly, the signaling of fibroblast growth factor (FGF) through its receptor (FGFR) tyrosine kinase is dependent on cell surface heparan-sulfate that connects individual FGF–FGFR complexes to yield an active signaling platform (33, 34). In a similar manner, APRIL cross-linked by proteoglycans could be important in mediating the survival of syndecan- and BCMA-positive plasma cells. Although our attempts to activate APRIL with heparin had limited success, it is known that the fine structures of heparin and the glycosaminoglycan side chains of proteoglycans are quite different and heparin is therefore not necessarily expected to mimic cell surface proteoglycans (35). Alternative hypotheses regarding the active form of APRIL exist. For example, a fraction of endogenous APRIL may remain membrane bound in the form of a chimeric protein formed as a result of alternative splicing between the closely located genes for TWEAK and APRIL (36).

Not only do heparansulfates modulate the activity of binding partners by cross-linking or inducing conformational changes, but they are also used for the generation of chemotactic gradients. The basis of chemotaxis for most chemokines relies on their concentration-dependent binding to cell surfaces or matrix heparansulfates (27, 37). Therefore, it is an intriguing possibility that heparansulfate-bound APRIL not only regulates plasma cell survival but also trafficking. Our observation that APRIL, either alone or in conjunction with BAFF, is important for the bone marrow tropism of newly generated plasma cells (and/or for their survival in this location) would agree with this hypothesis. Alternative interpretations are, however, possible: for instance, APRIL may induce upregulation of chemokine receptors that, in turn, would favor migration to the bone marrow.

Multiple myeloma and various leukemias rely, at least in part, on autocrine antiapoptotic signals delivered by APRIL and BAFF (38–41). Moreover, mice that are transgenic for APRIL develop lymphoid tumors that are derived from the peritoneal B-1 B cell population (42). Because APRIL alone displays little or no biological activity, only cell-bound APRIL may exert its oncogenic effects via TACI and/or BCMA, both of which are activators of the antiapoptotic NF–κB pathway (1). Proteoglycans are well-known tumor markers that can be either up- or down-regulated (35, 43, 44). For example, the tumor-specific splice variants of CD44 carry, among other features, a heparansulfate side chain at-
tached to the variant exon 3 that is absent in the standard form of CD44 (45). Both syndecan-1 and CD44 variants are expressed in myeloma and, in addition to binding growth factors, promote adhesion to bone marrow stromal cells that become stimulated for IL-6 secretion (43, 46, 47). IL-6 acts as a survival factor for myeloma cells, and its action is synergized by BAFF and APRIL (38, 41). Hematopoietic cells expressing proteoglycans could thus accumulate APRIL, rendering it active for TACI and/or BCMA signaling and triggering autocrine growth and tumorgenesis. APRIL has also been shown to stimulate the proliferation of tumor cells that lack TACI and BCMA. However, compared with the B cell costimulatory activity, this effect is marginal. It may still be that APRIL induces survival directly through syndecans that can deliver signals through their intracellular tails upon binding to ligands (48), which may explain the observation that APRIL H98 failed to stimulated tumor cell growth (unpublished data). In any case, the inhibition of APRIL by BCMA:Fc or specific other inhibitors that interfere with APRIL should be considered in cancer therapy.

MATERIALS AND METHODS

Cell lines and reagents. HEK-293T, NIH-3T3, SW480, A549, and HT29 cells were grown in DMEM with 10% FCS plus antibiotics. Where indicated, 50 mM sodium chlorate was added in culture medium for 4 d before the analysis. Jurkat, U266, BJAB, and IM9 cells were grown in RPMI 1640 with 10% FCS plus antibiotics. Heparin (Liquemin, 5,000 IU/ml) was purchased from Roche Pharma, and heparin-Sepharose was purchased from GE Healthcare.

Expression constructs. Expression vectors for Flag ligands and Fc:ligands have been described previously (17). Ligands were cloned either with a Flag or an Fc tag (amino acid numbers are given in parentheses): hBAFF-A134 (134–285); hBAFF-G137 (137–285); hBAFF-V142 (142–285); hAPRIL-A88 (88–233); hAPRIL-A88 (88–233 with mutations R129S/R172S/H203E); hAPRIL-Q92 (92–233); mAPRIL-H98 (98–233); mAPRIL-A88 (88–232); mAPRIL-H98 (98–232); mBAFF (127–309); mCD40L (115–260); mED1A (245–391); and the fusion proteins human hAPRIL (88–96)–hBAFF (142–285) and hBAFF (134–142)–hAPRIL (98–233). The expression vector for full-length APRIL has been described previously (8).

The extracellular domains of hBCMA (2–54), hTACI (2–159), and hCD40 (1–193) were also expressed as fusion proteins with the pentamerization domain of human cartilage oligomeric matrix protein (hCOMP, aa 33–80) and a Flag tag (49). BCMA:Fc was isolated from these supernatants using heparin-Sepharose beads (for 16 h at 4°C). Beads were washed with PBS and eluted by boiling in an SDS-PAGE sample buffer. 1/20 of the eluate was analyzed by Western blotting with anti-Flag M2 mAb (Sigma-Aldrich) or anti-hAPRIL mAb (Apli-2). Cells were analyzed using a four-color FACSCalibur flow cytometer and CellQuest software.

Immunoprecipitations. The various Flag-tagged APRIL and BAFF proteins (1 ml of cell supernatant, ∼1 μg) were immunoprecipitated with either 1 μg BCMA:Fc, followed by protein A-Sepharose beads, or with 10 μl of heparin-Sepharose beads (for 16 h at 4°C). Beads were washed with PBS and eluted by boiling in an SDS-PAGE sample buffer. 1/20 of the eluate was analyzed by Western blotting with anti-Flag M2 mAb (Sigma-Aldrich) or anti-hAPRIL mAb (Apli-2). Cells were isolated from spleens, inguinal lymph nodes, or the blood of C57BL/6 mice by anti-B220 magnetic bead separation (Miltenyi Biotech). B cells (105 cells/well in 200 μl RPMI 1640 with 10% FCS and 5 mM 2-mercaptoethanol) were grown for 48 h with 5 μg/ml of goat F(ab’)2 anti-mouse μ chain antibody (Jackson Immunoresearch Laboratories) and in the presence of serial dilutions of various Flag-tagged ligands. The assay was performed in the presence or absence of anti-Flag antibody (1 μg/ml) or heparin (0.01 μg/ml, corresponding to ∼2.5 μg/ml). Cells were pulsed for an additional 18 h with 1 μCi/well of [3H]thymidine, harvested, and counted by liquid scintillation.

Immunizations and treatments with decay receptors. C57BL/6 mice used in this study were housed at the Biogen Idec animal facility under sterile, pathogen-free conditions according to the approved Institutional Animal Care and Use Committees’ protocol. 6–8-wk-old mice were immunized i.p. with 100 μg (4-hydroxy-3-nitrophenyl)acetyl (NP) conjugated to chicken γ-globulin (CGG) at 21:1 molar ratio (NP21-CGG conjugate; Biosearch Technologies) precipitated in alum (Pierce Chemical Co.). BCMA:Fc and BAFFR:Fc were described previously (50), and normal human IgG was used as a control (Jackson Immunoresearch Laboratories). 250 μg of either receptor was administered by i.p. injection 6 d after immunization. On day 11 after the immunization, the mice were killed to collect spleen, bone marrow, and sera.

Measure of the antibody response. The frequency of antigen-specific antibody-secreting cells was estimated by ELISPOT using mixed celluloce esters (HA) 96-well plates (Millipore) coated overnight at 4°C with 50 μg/ml NP167-BSA or NP167-BSA in PBS. Plates were washed twice with PBS and blocked for 2 h with culture medium before culture of 3 × 105 cells/well of splenocytes or bone marrow cells, for 20 h in DMEM with 5% FCS, and 0.1 mM 2-mercaptoethanol. The plates were washed and the spots were visualized using horseradish peroxidase–conjugated goat anti–mouse IgG1 (Southern Biotechnology Associates, Inc.) followed by 3-amino-9-ethyl-
carbazole substrate (AEC single step solution; Zymed Laboratories). The reaction was terminated by washing plates with water, and the spots were counted with the aid of a dissecting microscope.

ELISA plates coated with NP$_2$-BSA or NP$_2$-BSA were blocked, incubated with a serial dilution of sera starting at 1:10,000, and revealed with horseradish peroxidase–conjugated goat anti–mouse IgG followed by an incubation with 3,3′,5,5′-tetramethylbenzidine substrate and an absorbance measurement at 450 nm (1-step turbo TMB ELISA; Pierce Chemical Co.). Titors were normalized against the value obtained for a 1:50,000 dilution of a hyperimmunized mouse serum. This mouse had been immunized with 100 µg NP$_2$–CGG in alum, boosted at day 30 with 50 µg NP$_2$–CGG, and killed at day 60 to collect serum (51).

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