Selectivity of BAFF/BLyS and APRIL for Binding to the TNF Family Receptors BAFFR/BR3 and BCMA

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ABSTRACT: BAFF (B cell activating factor of the TNF family, also known as BlyS and TALL-1), a TNF family cytokine critical for the development and function of B cells, has been reported to bind to three receptors, BCMA (B cell maturation protein), TACI (transmembrane activator and CAML [calcium-modulator and cyclophilin ligand] interactor), and BAFFR (BAFF receptor), but with widely conflicting values for the affinity and selectivity of binding. BCMA and TACI additionally bind APRIL (a proliferation-inducing ligand), the TNF family ligand most homologous to BAFF. Using soluble, monomeric forms of the receptors, we demonstrate that BAFFR binds BAFF with K_D ~16 nM, while BCMA binds with K_D ~1.6 μM, indicating a ~100-fold selectivity for binding to BAFFR over BCMA. APRIL shows the opposite selectivity, binding to BCMA with K_D ~16 nM while showing no detectable affinity for BAFFR (K_D > 3 μM). The binding of BAFF or APRIL to these receptors is highly sensitive to assay-dependent avidity effects, likely explaining the widely ranging affinity values reported in the literature. Binding of BAFF to BCMA-Fc, a bivalent fusion protein consisting of the extracellular domain of BCMA fused to the hinge and CH1 and CH2 domains of human IgG1, in solution or coated onto an ELISA plate gave apparent binding affinities of ~0.63 and ~0.15 nM, respectively, compared to values of $K_{D(app)} \leq 30$ and ~100 pM for the corresponding BAFFR/IgG1 fusion protein, BAFFR-Fc. The high selectivity of BAFF for BAFFR versus BCMA is thus partly obscured in these multivalent assays. The intrinsically high selectivity inferred from the measurements with monomeric receptor correlates well with *in vivo* data from knockout mice, providing a possible explanation for the observations that interruption of the BAFFR gene in the A/WySnJ mouse produces a phenotype similar to the BAFF knockout mouse, while the BCMA knockout mouse has no discernible B cell phenotype.

BAFF[1] (also known as BLyS, THANK, TALL-1, zTNF4, and TNFSF13B) is a TNF family ligand tightly linked to the development and maturation of B cells (*1*−*4*). There are three candidate receptors for BAFF, the TNF receptor family proteins BCMA, TACI, and BAFFR (also know as BR3). Understanding the selectivity of BAFF for each of these receptors *in vivo* is critical for elucidating their respective biological functions. In addition, two of these receptors, BCMA and TACI (*5*−*8*), also bind to the homologous TNF family ligand APRIL, further complicating the elucidation of the biological roles of these receptors. BAFF and APRIL each contain three receptor-binding sites, and consequently, their interactions with their cell-surface receptors are multivalent. Achieving an understanding of the selectivities of BAFF and APRIL for their various proposed receptors therefore requires elucidation of the true 1:1 binding affinities of each ligand for each of its receptors, as well as investigation of the role of avidity effects in governing the nature and strength of the interactions that can form. Making quantitative measurements of binding affinities and stoichiometries for TNF family receptors and ligands is nontrivial. The majority of published studies on BAFF or APRIL binding to their candidate receptors utilize bivalent receptor-Fc fusion proteins. The interaction of trimeric BAFF or APRIL with bivalent receptor constructs results in an apparent affinity for binding that contains an avidity component of significant but unpredictable magnitude. Consequently, the literature contains widely disparate K_D values for these ligands binding to each of their candidate receptors and therefore for their potential selectivity among these receptors.

BAFF is expressed on myeloid cells as a type-II transmembrane protein that can be cleaved from the membrane by a furin-like convertase and released as a soluble ligand (*1*, *2*). The receptors for BAFF are primarily restricted to B cells, and BAFF is a key costimulatory molecule for mature B cell proliferation and survival. BAFF transgenic mice display symptoms of systemic lupus erythematosus (SLE) and Sjogren's syndrome, including B cell hyperplasia and elevated autoantibody production (*9*). Conversely, BAFF-

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[1] Abbreviations: APRIL, a proliferation-inducing ligand; BAFF, B cell-activating factor of the TNF family; BAFFR, BAFF receptor; BAFFR-Fc, fusion protein consisting of the extracellular domain of BAFFR and the CH1, CH2, and hinge region of human IgG1; BCMA, B cell maturation protein; BCMA-Fc, fusion protein consisting of the extracellular domain of BCMA and the CH1, CH2, and hinge region of human IgG1; CRD, cysteine-rich repeat domains; EC_{50}, the value at which 50% of the maximum response is attained; TACI, transmembrane activator and CAML (calcium-modulator and cyclophilin ligand) interactor.

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deficient mice have a severe reduction in the number of mature B cells, reduced serum immunoglobulin, and severely impaired humoral immune response to both T-dependent and T-independent antigens (*10*, *11*).

APRIL has the ability to stimulate tumor cell growth (*12*), and it also stimulates B cell proliferation (*8*). It has been reported that a fusion protein consisting of the extracellular domain of BCMA fused to the hinge, CH1 and CH2 domains of human IgG1 (BCMA-Fc) is able to block APRIL-induced tumor cell growth (*13*). APRIL-deficient mice show no detectable abnormality in development or in B- or T cell function (*14*). This result leaves open the question of the true role for APRIL in immune cell development and maintenance. However, BAFF/APRIL heterotrimers have been detected in the serum of patients with various autoimmune diseases (*15*) and in the culture medium from BAFF/APRIL cotransfected cells. Interestingly, TACI-Fc but not BCMA-Fc or BAFFR-Fc was able to block the *in vitro* B cell proliferation induced by these heterotrimers (*15*).

BCMA was originally identified by a genetic translocation event in a T cell tumor line and was later shown to be natively expressed primarily by mature B cells (*16*, *17*). Some reports have indicated that BCMA expression is limited to the Golgi (*18*), while others have reported cell-surface expression in transfected cell lines (*4*). Targeted gene deletion experiments in mice have shown that BCMA, like APRIL, is dispensable for B cell development and maintenance of the humoral immune response, indicating that BCMA is not the primary receptor responsible for BAFF activity *in vivo* (*19*).

BAFFR was identified as a receptor for BAFF in BAFF responsive systems where the other known BAFF receptors were not present. BAFFR was shown to bind uniquely to BAFF and not to APRIL (*20*, *21*). The A/WySnJ mouse strain displays a severe deficit in mature B cells (*22*) similar to that of BAFF-deficient mice. The mutation responsible for this phenotype has been identified as a disruption in the intracellular signaling domain of BAFFR, confirming the critical role of BAFFR in BAFF-mediated B cell maturation (*10*, *20*, *21*).

TACI, identified in a two-hybrid screen for proteins that interact with CAML (*23*), is expressed on B cells and activated T cells and may be required for immune system homeostasis. Knock-out experiments in mice demonstrated that TACI is a negative regulator of B cell activation. The TACI-deficient mouse phenotype is similar to the transgenic BAFF mouse, with symptoms of autoimmune antibody formation and B cell hyperplasia (*21*, *24*, *25*).

All of the candidate BAFF and APRIL receptors differ from the canonical structure of TNF receptor family members. Typically, TNF receptors contain 2−4 cysteine-rich repeat domains (CRD), and in those cases for which the intermolecular contacts have been defined either by X-ray crystallography (*26*−*29*) or site-directed mutagenesis (*30*−*34*), the ligand makes discrete contact with two adjacent CRD. BAFFR is the smallest known member of the TNF receptor family, containing only a partial CRD (*20*), while BCMA contains only one. TACI contains two CRD, but they are of atypical structure (*35*). This structural difference requires that these receptors interact with their ligands in a manner that is distinct from the canonical mechanism. This expectation has been confirmed by structural studies of the complexes of BAFF with BCMA and with BAFFR. The canonical binding mode for TNF family receptors is along a groove formed at the monomer−monomer interface of the ligand, parallel to the 3-fold axis of symmetry, where two CRD from each receptor interact with two distinct patches on the ligand composed of approximately equal contributions from each monomer (*26*−*29*). BAFFR however binds to a small surface cavity in BAFF, formed primarily by residues from a single monomer with minor contribution from the adjacent monomer (*36*−*39*). BCMA appears to bind at an overlapping but nonidentical region that is restricted to a single monomer (*37*). Modeling the BCMA−APRIL complex, based on the crystal structures of APRIL (*40*) and the BAFF−BCMA complex (*37*), indicates that BCMA fits into a hydrophobic pocket that is conserved between BAFF and APRIL and that the binding modes are likely similar for BCMA binding to these two ligands (*40*). Because of the widely varying values reported for the interaction affinities of BAFF with its receptors, it is unclear whether the fact that BAFFR and BCMA are smaller than typical TNF-R family members and interact with their ligands in a different way necessarily translates to a lower interaction energy or whether nature somehow manages to engineer the same binding strength into a smaller contact surface.

Here, we characterize the binding of mono- and bivalent forms of the receptors BCMA and BAFFR to BAFF and APRIL. Using soluble, mono- and bivalent forms of these candidate receptors allows us to quantitatively assess the affinity of receptor binding to each ligand and to distinguish affinity from avidity contributions to binding inherent in bivalent receptor constructs. Our results demonstrate that BAFF displays an approximately 100-fold selectivity for binding to monomeric BAFFR over monomeric BCMA, while APRIL shows the opposite selectivity, binding with high affinity to BCMA while showing no binding to BAFFR whether presented as a monomer or dimer. When multivalent forms of the receptor are used, a substantial additional contribution to binding is seen because of avidity effects. The avidity contribution of dimeric versus monomeric BCMA results in >1000-fold increase in the apparent affinity for binding to BAFF, while the apparent K_D for dimeric BAFFR binding to BAFF is similarly increased by a factor of >500 over monomeric BAFFR. For technical reasons, these assay-dependent avidity effects partly obscure the high intrinsic selectivity of the interactions. Thus, although the interactions of BAFF with its receptors are presumably multivalent *in vivo*, it is necessary to work with well-characterized, monovalent receptor constructs to gain an accurate picture of binding selectivity in this system. Our results potentially provide a molecular basis for understanding the different phenotypes of the BCMA- and BAFFR-deficient mouse strains and thus provide clues to unraveling the complexity of the functions of BAFF and its receptors.

MATERIALS AND METHODS

myc-Human BAFF. Purification of BAFF has been described (*4*). Briefly, myc-hBAFF (Gln136-Leu285) was expressed in *Pichia pastoris*, and the supernatant was buffer-exchanged to 10 mM Tris-HCl at pH 7.2 and 50 mM NaCl and then loaded into a Q column and eluted with a salt gradient (50−500 mM NaCl). Further purification of myc-hBAFF was achieved by size-exclusion chromatography

on Superdex 75 (26/70) followed by Superdex 200 (26/70) columns. The purified protein was analyzed by SDS−PAGE followed by Coomassie Blue staining, Western blot analysis using mouse monoclonal 9E10 (anti-myc) antibody, and by N-terminal sequencing. Molecular weight was determined by mass spectrometry. Protein concentration was determined by absorbance at 280 nm using an extinction coefficient calculated from the amino acid sequence.

Human APRIL. Conditioned medium containing FLAG-tagged human APRIL from 293T cell cultures was brought to a final concentration of 10 mM $CaCl_2$ and 150 mM NaCl and filtered. The medium was loaded onto an M1 (anti-FLAG antibody) Sepharose affinity column (Sigma−Aldrich Corp., St. Louis, MO) 3 times by gravity at 4 °C. The column was then washed with 10 column volumes of 50 mM Tris at pH 7.6, 150 mM NaCl, and 1 mM $CaCl_2$. FLAG-tagged human APRIL was eluted from the column with 5 column volumes of 50 mM Tris at pH 7.6 and 150 mM NaCl containing 100 μg/mL FLAG peptide (Sigma−Aldrich Corp.). The eluted fractions were analyzed for purity by SDS−PAGE, and fractions containing human APRIL were dialyzed overnight into 20 mM Tris at pH 6.8 and then loaded onto a HiTrap SP column (Pharmacia, Piscataway, NJ). The column was washed with 4 column volumes of 20 mM Tris at pH 6.8. FLAG-tagged human APRIL was eluted in a single step with 1 M NaCl in 20 mM Tris at pH 6.8. The protein was further purified using a Superdex 200, 10 mm × 300 cm gel-filtration column (Pharmacia). Analysis by nonreducing SDS−PAGE revealed two closely spaced bands of 21 and 22 kDa, most likely representing glycosylated and nonglycosylated forms of the protein. Protein concentration was determined by absorbance at 280 nm using an extinction coefficient calculated from the amino acid sequence.

BCMA-Fc and Monovalent BCMA-Fc. The extracellular domain of BCMA (Met1-Gly51) was fused with human IgG1 Fc and stably transfected into CHO cells (*4*). The protein was purified using Protein-A-affinity chromatography followed by size-exclusion chromatography. To obtain monomeric BCMA-Fc, hydrophobic interaction chromatography using a resource phenyl column was performed using 225 mM $(NH_4)_2SO_4$ plus 50 mM phosphate. The protein was eluted with one-step 20 mM phosphate. Appropriate fractions were selected for SDS−PAGE under reducing and nonreducing conditions. Identification of bi- and (partially cleaved) monovalent BCMA-Fc was confirmed by the N-terminal sequence. Protein concentration was determined by absorbance at 280 nm using an extinction coefficient calculated from the amino acid sequence.

BAFFR-Fc. Construction and purification of BAFFR-Fc has been described (*4*). Briefly, the extracellular domain of BAFFR (Asp2-Ala71) was fused to human IgG1 Fc and stably transfected into CHO cells. The protein was purified using Protein-A-affinity chromatography followed by size-exclusion chromatography. Protein concentration was determined by absorbance at 280 nm using an extinction coefficient calculated from the amino acid sequence.

Monomeric BCMA and BAFF-R. Monomeric BCMA and BAFFR were obtained by controlled digestion of BCMA-Fc and BAFFR-Fc, respectively, with endoproteinase Lys-C. After cleavage, the Fc fragments were removed by Protein-A-affinity chromatography and the monomeric BCMA and BAFFR were further purified by size-exclusion chromatography. The purity of the proteins was analyzed by SDS−PAGE, and molecular weight was determined by mass spectrometry. Protein concentration was determined by amino acid analysis.

ELISA. Microtiter plates were coated overnight in PBS with either BAFF or APRIL (R&D Systems, Minneapolis, MN) at 2.5 μg/mL or with biotinylated BCMA or BAFFR at 10 μg/mL and blocked for an hour at room temperature in 1% BSA in PBS. Biotinylated ligands were added to plates containing immobilized receptor or biotinylated receptor were added to plates containing immobilized ligand for 30 min at 37 °C. Bound biotinylated ligand or receptor was detected using Streptavidin-HRP. Plates were developed for 5 min at ambient temperature with 100 μL of TMB Microwell peroxidase substrate (Kirkegaard and Perry laboratories). The reaction was stopped using 2N H_2SO_4, and plates were read at 450 nm. EC_{50} values were determined using an empirical four-parameter fit to the data

$$y = (a((bx)^c)/(1 + (bx)^c)) + d$$

where a = upper asymptote, b = midpoint (EC_{50}), c = slope of the linear part of the curve, and d = lower asymptote.

Biacore Surface Preparation. All experiments were performed using a Biacore 3000 instrument (Biacore AB, Uppsala, Sweden). BCMA-Fc, BAFFR-Fc, or BAFF were immobilized on CM5 sensorchips using the Biacore Amine Coupling Kit according to the instructions of the manufacturer. Briefly, proteins were diluted to 50 μg/mL in 10 mM acetate at pH 4.5, and 50 μL was injected over chip surfaces that had been activated with a 50 μL injection of 1:1 *N*-hydroxsuccinimide (NHS):1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC). Excess free amine groups were then capped with a 50 μL injection of 1 M ethanolamine. BCMA-Fc and BAFFR-Fc chip surfaces were conditioned with 5 × 30 s injections of 10 mM NaH_2PO_4. Typical immobilization levels for both BCMA-Fc and BAFFR-Fc were 4000−6000 RU. BAFF surfaces were conditioned with 5 × 30 s injections of biacore buffer (10 mM HEPES at pH 7, 150 mM NaCl, 3.4 mM EDTA, 0.005% p-20 (detergent), and 0.1% BSA). Typical immobilization levels for BAFF were 3000−4000 RU. All samples were prepared in biacore buffer. This same buffer was used as the running buffer during sample analysis. For immobilizations, the same biacore buffer without BSA was used.

Solid-Phase Biacore Binding Assays. Monomeric receptors were diluted in biacore buffer to the indicated concentrations and injected over the BAFF-derivatized surface or over an underivatized surface as a background control at a flow rate of 50 μL/min. The BAFF surface could not withstand regeneration, and the ligand was instead removed by long-term (10 min) dissociation. Bound ligand was completely removed in this time frame, and the signal returned to the baseline (see parts A and B of Figure 5). In all cases, binding to the blank chip was negligible. Affinity was determined by fitting plots of the response value where binding has come to equilibrium (R_{eq}) versus the concentration to a hyperbolic binding equation or by global fitting of all concentration sensorgrams to the kinetic model shown below (*42*) for simultaneously determining the on and off rates within the BIAevaluation software

$$dAB/dt = (k_a AB) - (k_d AB)$$

$$dB/dt = -((k_a AB) - (k_d AB))$$

where A = concentration, $B[0] = R_{max}$, and $AB[0] = 0$.

Solution-Phase Biacore Binding Assays. BAFF and the receptor were mixed in various ratios in biacore buffer and incubated at 4 °C for a minimum of 3 h. From each of these solutions, 150 μL was then passed at 10 μL/min over a BCMA-Fc-derivatized surface or over an underivatized surface as a background control, followed by dissociation for 3 min in biacore buffer. The surface was regenerated with 2 × 20 μL injections of 10 mM NaH_2PO_4 following each sample injection. Using these experimental conditions, the binding is mass-transport-limited during approximately the first minute of binding. The mass-transport-limited region was determined from the region of the sensorgrams where the slope of the first derivative plot is zero. During the mass-transport-limited binding phase, the initial rate of binding (V_i) is proportional to the concentration of free ligand in solution. Because only free BAFF is able to bind to the BCMA-Fc surface, this method measures the concentration of free BAFF in each solution. The affinity and stoichiometry of the interaction of BAFF and its receptors in solution was determined by plotting V_i versus the concentration of the varied component in each mixture and fitting the data to the following quadratic equation (*41*) that has the K_D and stoichiometric ratio (n) as variables:

$$V_i/m = [\text{BAFF}]_f = 1/2([\text{BAFF}]_t - \{(n[\text{R}]_t + [\text{BAFF}]_t + K_D) - \sqrt{[(n[\text{R}]_t + [\text{BAFF}]_t + K_D)^2 - (4n[\text{R}][\text{BAFF}]_t)]}\})$$

where V_i = initial rate of binding, m = slope of the BAFF standard curve, $[\text{BAFF}]_f$ = free BAFF concentration, $[\text{BAFF}]_t$ = total BAFF concentration, and $[R]_t$ = total receptor concentration.

Proliferation Assay. The proliferation assay has been described (*4*, *20*). Briefly, mouse splenic B cells were isolated from 2 month old C57 black 6 mice using B cell recovery columns (Cedarlane Laboratories, Ontario, Canada). B cells were incubated in flat-bottom 96-well plates at 10^5 cells/well in 100 μL of RPMI supplemented with 10% FBS for 48 h in the presence of 5 μg/mL of $F(ab')_2$ fragment goat anti-mouse IgM antibody (Jackson ImmunoResearch Laboratories, PA) and 75 ng/mL BAFF or APRIL. Indicated concentrations of receptor were added to block the proliferation induced by the indicated ligand. Cells were pulsed for an additional 18 h with 1 μCi/well [^{3}H]thymidine and harvested. [^{3}H]Thymidine incorporation was monitored by liquid scintillation counting. IC_{50} values were determined by fitting the data to an empirical four-parameter equation as described above for the ELISA.

RESULTS

Initial studies of BAFF binding to its receptors BCMA and BAFFR were carried out by ELISA and Biacore using receptor-Fc fusion proteins. Results from ELISA (Figure 1) indicated that BAFF bound to immobilized BCMA-Fc and BAFFR-Fc with similar high affinities. The EC_{50} values determined by applying a four-parameter curve fit to the ELISA data are 0.15 and 0.1 nM, respectively. These EC_{50} values do not correspond to true affinity measurements. In each case, both of the binding partners are potentially multivalent, and thus, the strength of the binding likely includes a substantial avidity contribution. Moreover, the fact that one component is immobilized on a microtiter plate will lead to additional surface avidity effects if some BAFF molecules can become bound simultaneously to two or more nearby molecules of receptor-Fc on the assay plate surface. Consistent with the expectation of complex binding mechanisms of this kind, the data in Figure 1 do not conform to a simple hyperbolic binding model and instead were fitted using an empirical four-parameter equation. An additional limitation of the above method is that the data provide no direct insight into the stoichiometry of the receptor−ligand complex formed.

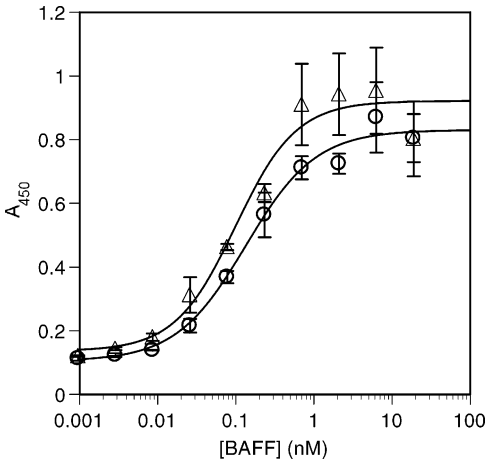

FIGURE 1: BAFF binding to immobilized receptor-Fc fusion proteins. The indicated concentrations of biotinylated BAFF were added to 10 μg/mL immobilized BCMA-Fc (○) or 10 μg/mL immobilized BAFFR-Fc (△) in PBS at pH 7 for 30 min at 37 °C. Bound ligand was detected with streptavidin-HRP as described in the Materials and Methods. Data points represent the mean of triplicate measurements. EC_{50} values were determined from a four-parameter curve fit (—) as described in the Materials and Methods. The data shown are representative of three independent experiments.

To overcome these limitations, we used a Biacore-based solution-phase binding method, which takes advantage of the fact that under conditions of so-called "mass-transport-limited" binding the initial rate of ligand binding is proportional to the concentration of ligand in solution (*42*). This is because, under these conditions, typically obtained at high receptor densities on the chip and low flow rates, binding of the soluble analyte to the immobilized receptor is fast compared to the diffusion of the analyte into the dextran matrix within which binding occurs. Consequently, the rate at which analyte binds to the chip is governed by the diffusion properties of the analyte and by the analyte concentration in the solution flowing across the chip surface and not by the kinetics of analyte−ligand binding. Figure 2A shows sensorgrams for a series of solutions containing BAFF at a range of concentrations binding to a BCMA-Fc chip under mass-transport-limited conditions. The initial rate of binding is determined over the first few seconds of association, as indicated by the linear fit to the initial binding phase of the sensorgrams shown as the red lines in Figure 2A. A plot of the initial velocity versus the concentration of BAFF (□, Figure 2B) shows that the initial velocity is indeed

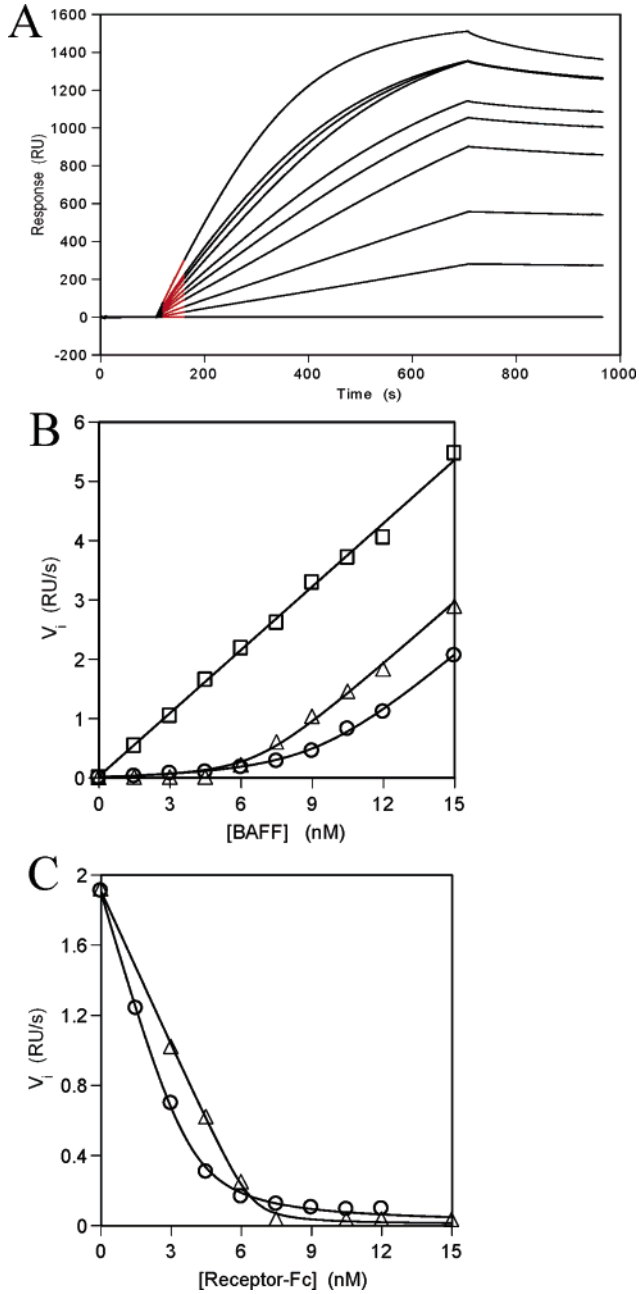

FIGURE 2: Solution-phase binding of BAFF to receptor-Fc fusion proteins. (A) Typical sensorgrams for 0−15 nM BAFF binding to a BCMA-Fc-derivatized Biacore chip. Initial rate data shown in B and C were determined over the first minute of binding, from ~150 to ~210 s as described in the Materials and Methods, and shown as linear fits (red lines) to the early region of the sensorgrams (black lines). (B) Separate solutions containing the indicated concentration of BAFF alone (□), BAFF plus 6 nM BCMA-Fc (○), or BAFF plus 6 nM BAFFR-Fc (△) were preincubated for 3 h and then run over a BCMA-Fc-derivatized Biacore chip as described in the Materials and Methods. The concentration of free BAFF in solution can be determined by reference to the standard curve. (C) Separate solutions containing the indicated concentration of BCMA-Fc plus 6 nM BAFF (○) or BAFFR-Fc plus 6 nM BAFF (△) were preincubated for 3 h and then run over a BCMA-Fc-derivatized Biacore chip as described in the Materials and Methods. The affinities and stoichiometries of the solution-phase binding of receptor-Fc fusion protein to BAFF were determined by fitting the data to the appropriate quadratic equation (—) as described in the Materials and Methods.

proportional to the BAFF concentration and, consequently, that the method can be used to obtain a rapid and accurate measure of the concentration of free BAFF present in solution. Figure 2B also shows the results obtained when a set of solutions containing various concentrations of BAFF were preincubated to equilibrium with a fixed concentration of 6 nM BCMA-Fc (○) or 6 nM BAFFR-Fc (△) before being run over by the BCMA-Fc-derivatized chip under identical conditions to those used in Figure 2A. In Figure 2B, at low concentrations of BAFF, the initial velocity is depressed relative to the standard curve obtained with BAFF alone, because most of the BAFF present is bound to receptor-Fc in the mixtures and is consequently not available to bind to the receptor-Fc on the chip surface. However, as the BAFF concentration is increased to the point where it reaches and then exceeds the concentration of available receptor-binding sites present in the preincubated solutions, V_i increases until it becomes parallel to the standard curve, indicating that the fixed amount of receptor-Fc in the solutions is fully occupied and each additional equivalent of BAFF thus remains free. The data for the BAFF/BCMA-Fc and BAFF/BAFFR-Fc mixtures can be fitted using a quadratic binding equation (see the Materials and Methods), to determine the affinity of the interaction between the BAFF and the receptor-Fc in the preincubated solutions and also the stoichiometry of the complex that is formed when they interact. Importantly, the measurement shown in Figure 2B is not a competition experiment; the average contact time of each volume of the flowing solution with the chip surface is only ~250 ms, and therefore, except for exceptionally weak complexes that have dissociation half-lives of <1 s, there is no opportunity for the BCMA-Fc on the chip surface to displace BCMA-Fc or BAFFR-Fc from a soluble complex. Rather, the method provides a rapid snapshot of the distribution of bound versus free BAFF present in each of the preincubated solutions, and thus, the affinity and stoichiometries obtained from the data represent true solution values. The data in Figure 2B show that BCMA-Fc and BAFFR-Fc can each bind to BAFF in solution to form a 1:1 complex. The slight differences in the titration end points arise from uncertainties in the protein concentrations of the components in the mixture. The apparent affinities obtained from the curve fits are 520 ± 160 pM for BCMA-Fc ($n = 5$) and ≤30 pM for BAFFR-Fc ($n = 6$), showing that both of these receptor-Fc constructs bind to BAFF with high affinity. These binding titrations can also be carried out in the reverse format, by holding the BAFF concentration constant and varying the concentration of receptor-Fc, as shown in Figure 2C. In this format, the initial velocity decreases with increasing receptor-Fc, as greater fractions of the BAFF become sequestered in complexes. Applying a similar quadratic binding equation to these data gave similar affinities for the interactions of BAFF with BCMA-Fc (770 ± 440 pM; $n = 4$) and BAFFR-Fc (≤30 pM; $n = 6$) to those obtained in Figure 2B. These results are collected in Table 1 and reported as the average of all titrations done in both formats.

While the method described above provides solution-phase affinity and stoichiometry data, the affinities measured in parts B and C of Figure 2 still represent apparent affinities rather than true K_D values. This is because the receptor-Fc proteins are dimeric constructs, each containing two copies of the receptor extracellular domain. This bivalency gives rise to an avidity component to the binding with the potentially trimeric BAFF, from which the true affinity of a

Table 1: Equilibrium Binding Measurements

	BAFF			APRIL		
	Biacore		ELISA	Biacore		ELISA
	solution binding (nM)[a]	immobilized BAFF (nM)[a]	immobilized receptor (nM)	solution binding (nM)	immobilized APRIL (nM)[a]	immobilized receptor (nM)
BAFFR	4.9 ± 2.9 ($n = 3$)	16 ± 6[b] ($n = 11$)	nd[c]	no binding	$\gg 3000$	no binding
BCMA	1400 ± 100 ($n = 3$)	1550 ± 150[b] ($n = 9$)	nd[c]	nd[c]	16 ± 8 ($n = 6$)	nd[c]
BAFFR-Fc	≤ 0.03[e] ($n = 12$)	N/A[d]	0.1[e] ($n = 1$)	no binding	N/A[d]	no binding
BCMA-Fc	0.63 ± 0.32[e] ($n = 9$)	N/A[d]	0.15[e] ($n = 1$)	nd[c]	N/A[d]	nd[c]

[a] Uncertainty limits are standard deviation. [b] K_D calculated from replot of R_{eq} versus [receptor] fitted to hyperbolic binding equation. [c] nd = not determined. [d] N/A = not applicable. [e] Apparent affinity, not a true K_D.

single receptor-binding site for a single receptor cannot easily be extracted. To overcome this limitation, we purified a version of BCMA-Fc in which one of the BCMA domains had been proteolytically cleaved from the Fc portion of the molecule, leaving just a single copy of BCMA attached to the Fc ("monovalent BCMA-Fc"). Parts A and B of Figure 3 show data from an ELISA-format assay in which biotinylated BAFF binds to mono- or intact bivalent BCMA-Fc immobilized on a microtiter plate. Figure 3A shows that when the mono- or bivalent BCMA-Fc is coated on the ELISA plate at a coating concentration of 1 μg/mL BAFF binds very poorly to the monovalent receptor construct in comparison to bivalent BCMA-Fc. Figure 3B shows that coating monovalent BCMA-Fc at a 10-fold higher concentration restores high-affinity binding to a level that is comparable to that for bivalent BCMA-Fc, presumably because at a high coating density the individual molecules of monovalent BCMA-Fc are typically close enough together on the assay plate surface that BAFF molecules can bind to multiple BCMA-binding domains as they do when interacting with the bivalent BCMA construct. The fact that strong binding was restored by coating the monovalent BCMA-Fc at a high density indicates that partial proteolysis of bivalent BCMA-Fc did not damage the remaining BCMA domain. The integrity of this reagent was also confirmed by N-terminal sequencing. To obtain an accurate value for the affinity of BAFF for monomeric BCMA, solution-phase Biacore binding experiments similar to those shown in Figure 2C were performed with monovalent BCMA-Fc. Figure 3C shows monovalent BCMA-Fc titrated in the presence of 500 nM BAFF. The binding data fit quite well to a hyperbolic, single-site binding model, indicating that the dissociation constant for the monovalent receptor is high relative to the fixed concentration of BAFF. The curve fit returned a value of $K_D = 1.4 \pm 0.3$ μM ($n = 2$). Thus, the monovalent BCMA-Fc binds to BAFF with an affinity that is nearly 3 orders of magnitude lower than that measured for bivalent BCMA-Fc (Table 1). As a result of the hyperbolic form of the binding curve in Figure 3C, it was not possible to determine the stoichiometry of the complex formed in this experiment.

To ensure that the weak binding that we observed with monovalent BCMA-Fc was not an artifact of having the bulky Fc domain attached to the small receptor domain, we made soluble monomeric BCMA by enzymatically cleaving the receptor domains from the Fc domain of the fusion protein. Monomeric BAFFR was made by a similar method. The sequences of the monomeric receptors were confirmed by mass spectrometry and N-terminal sequencing, to establish that the proteolysis conditions used in their preparation had not resulted in any cleavage or truncation of the receptor domains (see the Materials and Methods). Solution-phase Biacore measurements were made with these monomeric receptors. Figure 4A shows that titration of monomeric BCMA against 500 nM BAFF gave an affinity of $K_D = 1.4 \pm 0.1$ μM ($n = 3$) for this interaction, in good agreement with the data obtained using the monomeric BCMA-Fc construct. However, similar experiments performed with monomeric BAFFR gave a quite different result. Figure 4B shows that titration of monomeric BAFFR against 25 nM BAFF gave an affinity of 4.9 ± 2.9 nM ($n = 3$), about 300-fold stronger than the binding of monomeric BCMA (Table 1).

To confirm the data obtained with the solution-phase Biacore method and to obtain information about the association and dissociation kinetics of binding, we also measured the binding of BAFF to monomeric BCMA and BAFFR using conventional Biacore kinetic methods. In these experiments, BAFF was immobilized on the surface of a Biacore CM5 chip and various concentrations of the monomeric receptors were run over the chip. Figure 5A shows that the binding and dissociation of monomeric BCMA to the BAFF-derivatized surface were too rapid to yield rate information; both were complete within the dead time of the instrument. However, a plot of the signal achieved at equilibrium (R_{eq}) versus the BCMA concentration, shown in the inset of Figure 5A, fitted well to a hyperbolic binding equation for a single-site binding model. The curve fit gave an affinity of 1.6 ± 0.2 μM ($n = 9$), in excellent agreement with the value obtained in Figure 4A for the same interaction taking place in solution (Table 1). For BAFFR binding to immobilized BAFF (Figure 5B), the binding and dissociation were slow enough to be clearly observable and the rate constants were determined by globally fitting a set of 10 concentration curves spanning the [BAFFR] range of 15−150 nM to the equation for a single-site binding model. In Figure 5B, the data are shown as black lines and the red lines are the global fit to the data. An affinity of $K_D = 14 \pm 5$ nM ($n = 13$) was determined from the ratio $K_D = k_d/k_a$ (Table 2). In addition, the R_{eq} values were plotted as a function of the BAFFR concentration (inset of Figure 5B) and the results were fitted to a hyperbolic binding equation, giving an affinity of $K_D = 16 \pm 6$ nM ($n = 11$, Table 1). This value is in excellent agreement with the value obtained by analysis of on and off rates and with the value obtained in Figure 4B for the same reaction taking place in solution (Tables 1 and 2).

In the presence of anti-IgM antibody, BAFF has been shown to costimulate B cell proliferation *in vitro* (*1*, *2*, *43*, *44*). The BAFF-induced proliferation can be blocked by BCMA-Fc (*4*, *45*, *46*) and by BAFFR-Fc (*20*, *21*). As additional confirmation of the selectivity of the monomeric receptors seen in the binding assays, we tested the ability of

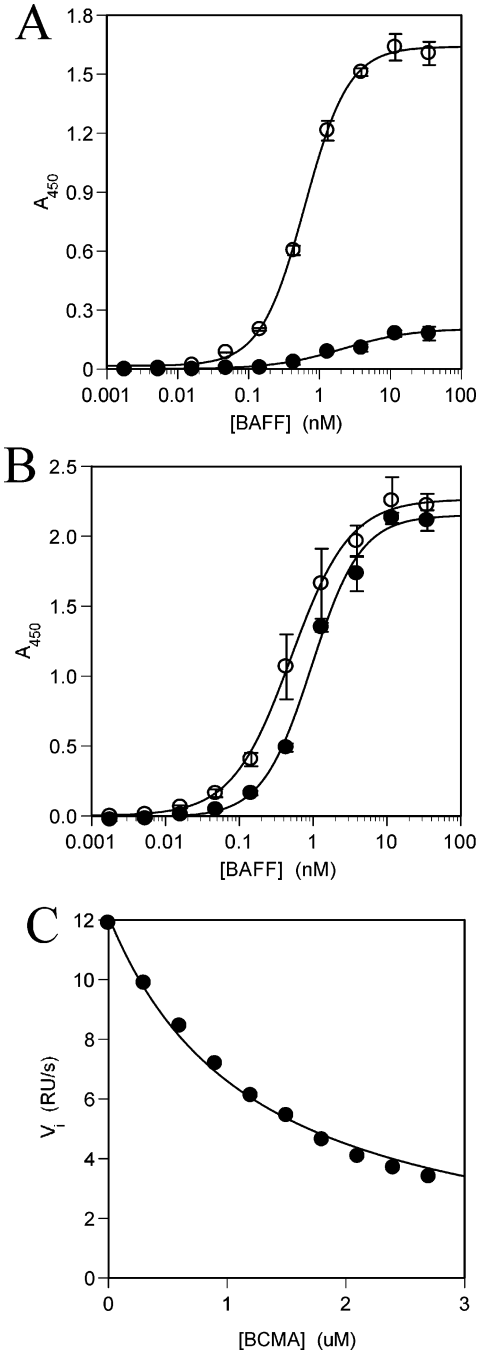

FIGURE 3: Bivalent BCMA-Fc versus monovalent BCMA-Fc binding to BAFF. (A) Binding of the indicated concentrations of biotinylated BAFF to ELISA plates coated with monomeric (●) or dimeric (○) BCMA-Fc at 1 μg/mL. (B) Binding of the indicated concentrations of biotinylated BAFF to ELISA plates coated with monomeric (●) or dimeric (○) BCMA-Fc at 10 μg/mL. In A and B, bound ligand was detected with streptavidin-HRP as described in the Materials and Methods. Data points represent the mean of triplicate measurements. The data shown are representative of three independent experiments. (C) BAFF (500 nM) was preincubated for 3 h with the indicated concentrations of monovalent BCMA-Fc. Solutions were then flowed over a BMCA-Fc-derivitized Biacore chip as described in the Materials and Methods. The affinity of the monovalent BCMA-Fc−BAFF interaction in solution was determined by fitting the data to a hyperbolic binding equation. Data are representative of four experiments.

monomeric BCMA or BAFFR to block BAFF-induced B cell proliferation. Figure 6 shows that monomeric BCMA had no effect on the proliferation of mouse splenic B cells induced by BAFF, even at concentrations similar to its K_D for binding to BAFF, while monomeric BAFFR was able to block BAFF-induced proliferation at concentrations consistent with its high affinity for BAFF. Figure 6 also shows that the higher affinity bivalent BCMA-Fc is able to block proliferation, but that bivalent BAFFR-Fc is 5−10-fold more effective as a blocking reagent. These results are consistent with previous findings (*47*), which established that BAFFR-Fc is more potent than BCMA-Fc as a blocker of BAFF activity in a variety of assay formats.

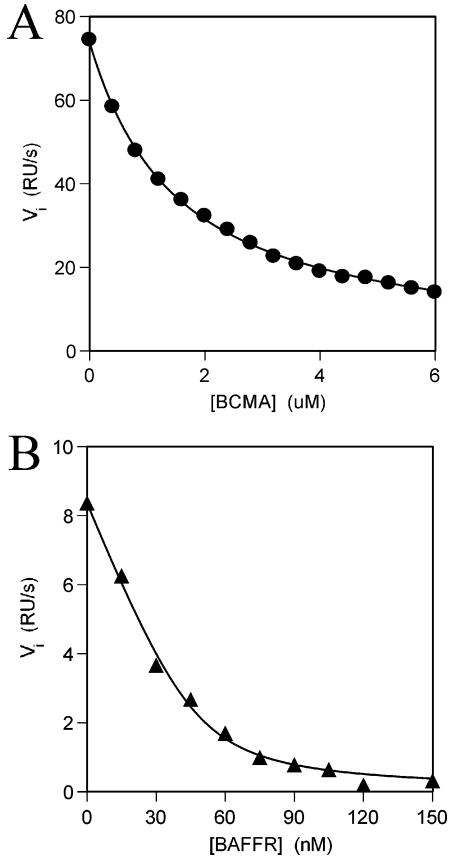

FIGURE 4: Solution-phase binding of BAFF and soluble, monomeric receptors. (A) BAFF (500 nM) was preincubated for 3 h with the indicated concentration of soluble, monomeric BCMA, or (B) 25 nM BAFF was preincubated for 3 h with the indicated concentration of soluble, monomeric BAFFR. Solutions were then run over a BCMA-Fc-derivatized chip as described in the Materials and Methods. The affinity of the solution-phase binding of BAFF with soluble, monomeric BCMA (●, A) or BAFF with soluble, monomeric BAFFR (▲, B) was determined by fitting the data to a quadratic binding equation (—) as described in the Materials and Methods. Data are representative of three experiments.

We also tested the ability of mono- and dimeric BCMA or BAFFR to bind to APRIL, the TNF family ligand most closely related to BAFF. Published studies have indicated that APRIL can bind to BCMA, although not to BAFFR (*20*, *21*). Estimates for the affinity of this interaction have been reported (*8*) but only using a bivalent receptor-Fc fusion protein. The ELISA results shown in Figure 7A demonstrate that, as expected, bivalent forms of both receptors and also monovalent BAFFR are able to bind to BAFF coated on an ELISA plate, while at the concentrations tested, no binding of monovalent BCMA was detected. In contrast, Figure 7B shows that both mono- and bivalent forms of BCMA are able to bind to APRIL coated on an ELISA plate, while at the concentrations tested (up to 500 nM), neither mono- nor bivalent forms of BAFFR showed any binding. These results

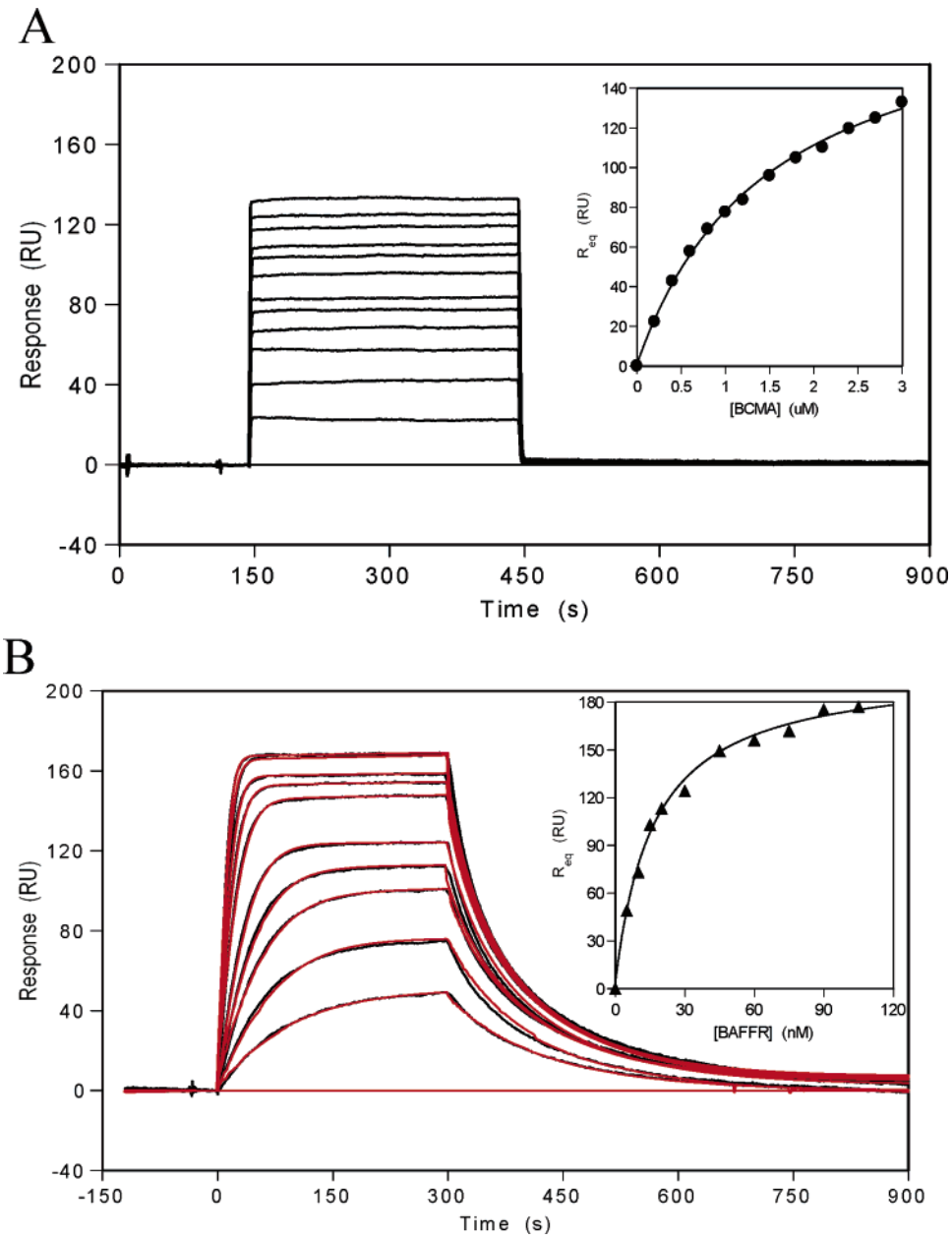

FIGURE 5: Soluble monomeric receptors binding to BAFF immobilized on a Biacore chip. (A) Sensorgrams for 0−3 μM soluble monomeric BCMA binding to BAFF immobilized on a Biacore chip. The association and dissociation of BCMA binding to immobilized BAFF are complete within the dead time of the instrument; therefore, no kinetic information is obtained. The affinity for soluble monomeric BCMA binding to immobilized BAFF was determined from a replot of the equilibrium response versus the concentration of monomeric BCMA (inset). The solid line is a fit to a hyperbolic binding equation (see the Materials and Methods). (B) Sensorgrams for 0−105 nM BAFFR binding to BAFF immobilized on a Biacore chip. The affinity of soluble monomeric BAFFR binding to immobilized BAFF was calculated from $K_D = k_d/k_a$ as determined using the rate equations within the BIAevaluation software as described in the Materials and Methods. The affinity for soluble monomeric BAFFR binding to immobilized BAFF was also determined from a replot of the equilibrium response versus the concentration of monomeric BAFFR (inset). The solid line is a fit to a hyperbolic binding equation (see the Materials and Methods). Data are representative of at least four experiments.

Table 2: Kinetic Binding Measurements

	BAFF		APRIL	
	BAFFR[a]	BCMA	BAFFR	BCMA[a]
k_a (M^{-1} s^{-1})	$1.4 \pm 1.5 \times 10^6$	$\geq 3.3 \times 10^5$	no binding	$5.1 \pm 2.9 \times 10^5$
k_d (s^{-1})	$1.3 \pm 0.8 \times 10^{-2}$	$>1 \times 10^{-1}$	no binding	$5.6 \pm 1.2 \times 10^{-3}$
K_D (nM)	14 ± 5^b ($n = 13$)	nd[c]	no binding	13.6 ± 8.6^b ($n = 4$)

[a] Uncertainty limits are standard deviation. [b] K_D calculated from the ratio k_d/k_a. [c] nd = not determined.

confirm previous reports that BAFFR is highly selective for binding to BAFF. In addition, they indicate that BCMA is a high-affinity receptor for APRIL.

To determine the precise affinities and selectivity of APRIL binding to BCMA and BAFFR, we measured the binding of both monomeric receptors to immobilized APRIL

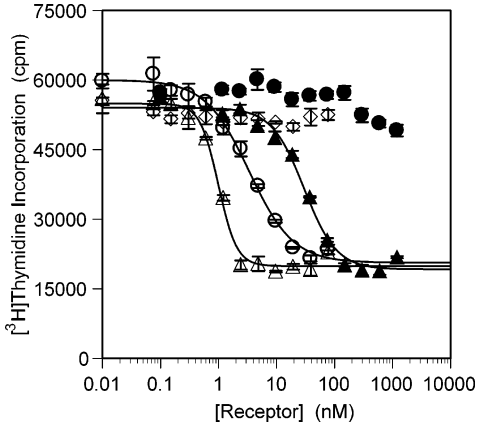

FIGURE 6: Soluble, monomeric receptors or bivalent receptor-Fc blocking BAFF-induced B cell proliferation. A total of 75 ng/mL BAFF plus 5 μg/mL anti-IgM F(ab′)$_2$ in the presence of the indicated concentrations of BCMA-Fc (○), BAFFR-Fc (△), monomeric BCMA (●), monomeric BAFFR (▲), or hIgG as a control (◇) were added to mouse splenic B cells as described in the Materials and Methods. B cell proliferation was measured by [^{3}H]-thymidine incorporation. Data points represent the mean of triplicate measurements. IC$_{50}$ values were determined from a four-parameter curve fit (—) as described in the Materials and Methods. The data are representative of three independent experiments.

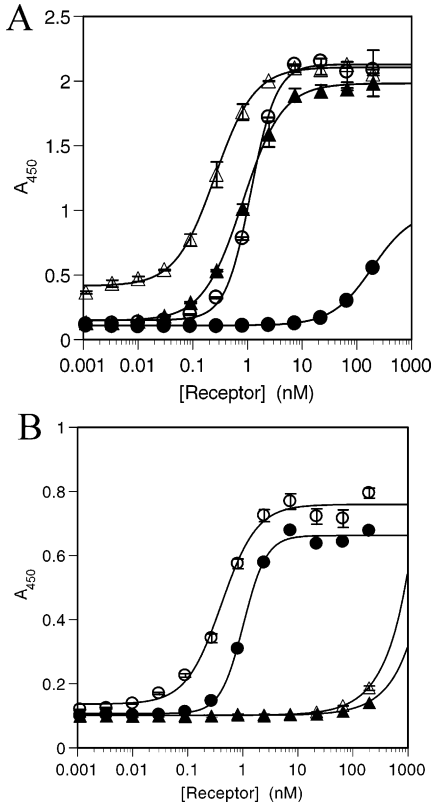

FIGURE 7: Soluble, monomeric receptors or bivalent receptor-Fc fusion proteins binding to BAFF- or APRIL-coated ELISA plates. The indicated concentrations of biotinylated BCMA-Fc (○), monomeric biotinylated BCMA (●), biotinylated BAFFR-Fc (△), or monomeric biotinylated BAFFR (▲) were added to BAFF-coated ELISA plates (A) or APRIL-coated ELISA plates (B) in PBS at pH 7 for 30 min at 37 °C. Bound receptor was detected with streptavidin-HRP as described in the Materials and Methods. Data points represent the mean of triplicate measurements and are fitted using a four-parameter equation (—) as described in the Materials and Methods. The data are representative of three independent experiments.

in Biacore experiments (Figure 8). Kinetic evaluation of the sensorgrams, performed as described above for BAFFR binding to BAFF in Figure 5B, indicated that the affinity of monomeric BCMA for APRIL is $K_D = 14 \pm 9$ nM ($n = 4$, Table 2). A plot of R_{eq} versus the BCMA concentration fitted to a hyperbolic binding equation gave a value for the affinity of this interaction of $K_D = 16 \pm 8$ nM ($n = 6$, Table 1), in very close agreement with the K_D obtained from the ratio of the kinetic rates, k_{off}/k_{on}. In similar experiments, monomeric BAFFR at concentrations up to 3 μM showed no interaction with immobilized APRIL, indicating that the K_D for this interaction, if it occurs at all, is much greater than 3 μM (Figure 8B). These results demonstrate that APRIL is selective for binding to BCMA over BAFFR by more than 100-fold. This observation was confirmed using the B cell proliferation assay shown in Figure 9, which shows that both mono- and dimeric forms of BCMA were able to block the APRIL-induced proliferation of mouse splenic B cells, while BAFFR had no effect either as a dimeric Fc-fusion protein or as a soluble monomeric receptor.

DISCUSSION

BAFF is a key mediator of humoral immunity through its role in B cell development and maturation (*1*, *2*, *48*, *49*, reviewed in ref *50*). Understanding the molecular mechanisms of action that lead to these effects is complicated by the fact that BAFF has been reported to engage in high-affinity interactions with three distinct receptors, BCMA, TACI, and BAFFR (*4*, *8*, *20*, *21*, *45*, *46*). Two of these receptors, BCMA and TACI, have been reported to bind also to APRIL, the TNF family ligand most homologous in sequence to BAFF (*8*, *13*). This plethora of potential interactions has made obtaining a precise molecular understanding of the roles of these ligands and receptors in immunity and disease a complex affair. In this paper, we have compared the ability of both BAFF and APRIL to bind to mono- and bivalent forms of two of these receptors, BCMA and BAFFR. Our goals were to obtain an unambiguous view of the selectivity governing the interactions among this set of ligands and receptors and to assess the magnitude of the avidity effects arising from using bivalent receptor constructs. We were unable to generate a nonaggregated form of TACI; therefore, this receptor was not included in these analyses.

Previous studies of the interactions of BAFF with its receptors have reported binding affinities ranging from 0.26 to 8000 nM for BCMA (*8*, *37*, *39*, *45*, *46*, *51*, *52*) and from 0.3 to 109 nM for BAFFR (*20*, *37*, *47*, *51*). With few exceptions, the affinity measurements were made using receptors expressed on the surface of transfected cells, captured at high density on an ELISA plate, or using bivalent receptor-Fc fusion proteins. In all such cases, the intrinsic selectivity of BAFF for interacting with the receptors is obscured by the large and potentially widely variable contributions of avidity effects to the strength of binding, arising from the multivalent nature of the interaction, making it difficult to draw quantitative conclusions. There are three exceptions to this caveat. In two papers by Liu et al., the binding of monomeric BCMA (*52*) or BAFFR (*37*) to BAFF was measured by Biacore. The authors report affinities of 113 and 109 nM, respectively, although very little experimental detail is provided. Patel et al. (*51*) found no

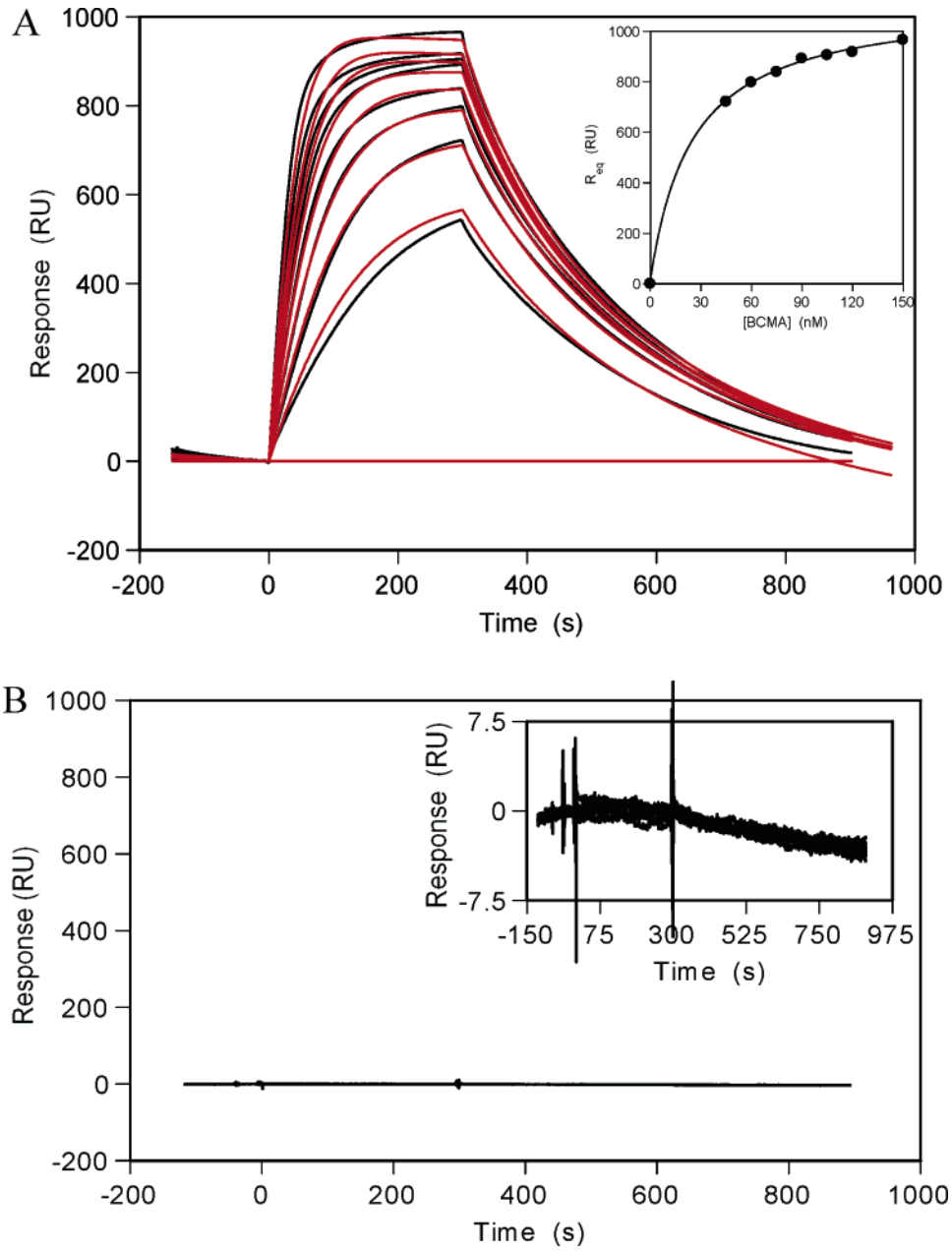

FIGURE 8: Soluble, monomeric BCMA or BAFFR binding to APRIL immobilized on a Biacore chip. (A) Sensorgrams for 0−150 nM BCMA binding to APRIL immobilized on a Biacore chip. The affinity of soluble monomeric BCMA binding to immobilized APRIL was calculated from $K_D = k_d/k_a$ as determined using the rate equations within the BIAevaluation software as described in the Materials and Methods. The affinity for soluble monomeric BCMA binding to immobilized APRIL was also determined from a replot of the equilibrium response versus the concentration of monomeric BCMA for the subset of sensorgrams that reached a plateau (inset). The solid line is a fit to a hyperbolic binding equation (see the Materials and Methods). Data are representative of four experiments. (B) Sensorgrams for 0−3 μM BAFFR binding to APRIL immobilized on a Biacore chip. Zooming in on the same data set (inset) shows no interaction between APRIL and BAFFR.

measurable binding of monomeric BCMA to BAFF by Biacore but obtained an IC_{50} value of 8 μM by competition ELISA.

In our experiments, we found that the measured affinities of BAFF for BCMA and BAFFR were highly sensitive to whether the receptor was presented captured on a surface, as a bivalent Fc fusion construct in solution, or as a soluble monomer. When BCMA-Fc or BAFFR-Fc were captured on the surface of a microtiter plate, BAFF was able to bind to both of these receptors with apparent affinities in the low nanomolar range, consistent with previous reports. Indeed, even a monovalent form of the intrinsically low-affinity receptor BCMA, when immobilized to high density on an assay plate, displayed high-affinity binding to BAFF. This relatively strong binding presumably results from the ability of BAFF to form simultaneous complexes with two or more neighboring receptor molecules. Even when measured in solution, both BCMA-Fc and BAFFR-Fc showed strong interactions with BAFF. When measured in these ways, no strong selectivity of BAFF for one receptor over another was evident, although binding to BAFFR-Fc appeared marginally stronger. Only when monomeric forms of the receptors were tested for their ability to bind to BAFF in solution did it become clear that BAFF binds to BAFFR with a much higher affinity than it does to BCMA (K_D ~16 nM versus 1.6 μM). The results obtained using monomeric BCMA and BAFFR

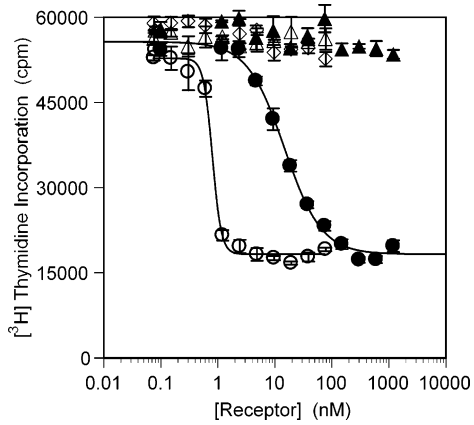

FIGURE 9: Soluble, monomeric receptors or bivalent receptor-Fc fusion proteins blocking APRIL-induced B cell proliferation. Indicated concentrations of BCMA-Fc (○), BAFFR-Fc (△), monomeric BCMA (●), monomeric BAFFR (▲), and hIgG as a control (◇) were tested for their ability to inhibit the B cell proliferation induced by 75 ng/mL of APRIL as measured by [^{3}H]thymidine incorporation. Data are from triplicate measurements made in a single experiment.

in solution (Figure 4) also demonstrate the absence of any strong allosteric coupling between the receptor-binding sites on BAFF. Any strong positive or negative cooperativity in the occupancy of the three binding sites on BAFF by successive receptor monomers would be expected to cause the binding curves to deviate from the single-site binding models shown to fit the data in Figure 4.

Several previous reports have shown that APRIL is able to bind with high affinity to BCMA (*8*, *13*, *46*, *51*). All of these reports utilize bivalent BCMA-Fc immobilized on either an ELISA plate, a Biacore chip, or in coimmunoprecipitation experiments. They are therefore subject to the caveats associated with using dimerized and/or immobilized receptors to quantify binding affinities to multivalent ligands, detailed above. Patel et al. (*51*) have also measured the binding of a monomeric form of BCMA to APRIL and report an affinity of ~5 nM, although the data were not shown and little experimental detail was provided. We performed binding measurements using monomeric BAFFR and BCMA to measure the affinities and selectivity of APRIL for binding to these two receptors. From ELISA, we have shown that both mono- and dimeric forms of BCMA bind to APRIL with high affinity, whereas neither mono- nor dimeric BAFFR were able to bind to APRIL even at receptor concentrations up to 200 nM. In kinetic experiments by Biacore (Figure 8A), similar to that shown for BAFF in Figure 5, we observed that APRIL bound monomeric BCMA with $K_D \sim 16$ nM, whereas monomeric BAFFR showed no detectable binding ($K_D \gg 3\ \mu$M) (Figure 8B). Taken together, these results indicate that BAFF has a high selectivity for binding to BAFFR over BCMA, while APRIL shows the opposite selectivity, binding to monomeric BCMA >100-fold more strongly than to BAFFR. The high selectivity of APRIL for BCMA versus BAFF was confirmed by showing that both mono- and dimeric forms of BCMA were able to inhibit APRIL-induced proliferation, while neither form of BAFFR had any effect. Conversely, mono- and dimeric BAFFR were able to block BAFF-induced proliferation, while monomeric BCMA was ineffective.

An additional feature of the "solution-phase" (initial rate) Biacore measurements is that they constitute a direct titration of ligand versus receptor. When the fixed component of the titration is present at a high concentration relative to the K_D, these measurements give a direct determination of the stoichiometry of the complex that forms in solution, provided that the concentrations of active ligand and receptor are accurately known. Measured in this way, the stoichiometries of the complexes formed in solution between BAFF and the receptor Fc fusions were 1:1 for both BAFFR-Fc and BCMA-Fc.

The selectivity of BAFF for binding to BAFFR versus BCMA observed with monomeric receptors, as compared to dimeric receptor-Fc fusion proteins, does not imply that binding is necessarily less selective when occurring in a multivalent context. On the contrary, one would expect that, all other things being equal, the overall selectivity for binding to BAFFR versus BCMA would increase with each additional interaction that forms within a multivalent complex. This is because occupancy of successive receptor-binding sites by BAFFR would each generate more binding energy than the corresponding interactions with the weaker-binding BCMA. The fact that this selectivity is less evident from the results obtained in the assays that allow multivalent binding is an artifact of the experimental methods rather than a mechanistic result. In each case, avidity effects cause binding to become so strong that it approaches or exceeds the measurement limits of the assay. Therefore, below a certain K_D, all ligands begin to look similarly potent. This is because, no matter how strong the binding, it is necessary to add at least 1 equivalent of ligand to titrate the receptor to full occupancy. The selectivity that we could demonstrate using these bivalent reagents either in solution- or solid-phase assays was therefore relatively modest (⩽20-fold), even though the true selectivity may have been much greater than even the factor of 100-fold seen using the monomeric receptor reagents. Evaluating the true selectivity of BAFF for bi- or multivalent forms of BAFFR and BCMA would require methods that could accurately measure affinities in the subpicomolar range. Equilibrium methods would require exceedingly high sensitivity because of the need to work with receptor and ligand concentrations at or below K_D as well as very long incubation times to achieve equilibrium. Kinetic methods, in which the K_D is calculated from on- and off-rate measurements, overcome some of these obstacles. Performing rigorous kinetic analyses of complex multivalent interactions, however, has difficulties of its own, and we did not attempt it in this case.

Avidity and surface effects of the kind described above presumably are responsible for the widely different values that have been reported for the affinities of these interactions when measured in different studies. Indeed, our data show that the apparent affinities measured using surface-captured receptor-Fc fusion proteins are somewhat arbitrary and can vary greatly with changes in the format of the assay (see parts A and B of Figure 2). *In vivo*, the receptor is expressed on membrane surfaces, allowing multiple receptor molecules to interact with each BAFF molecule. The multivalency of receptor−ligand binding that gives rise to these avidity effects is therefore certainly relevant to the biological mechanism of the receptor. However, the entropic situation that governs the avidity of binding for such membrane-expressed receptors, whether independently diffusing or existing as part of some specific preassociated complex, is

quite distinct from that of receptor molecules immobilized on a plastic assay plate or coupled to the dextran matrix of a Biacore chip (*53*). There is therefore no basis for assuming that such assays provide a quantitatively valid mimic of the interaction of endogenous BAFF with its cell-surface receptors *in vivo*. Indeed, no quantitative analysis of the role of avidity effects in BAFF signaling is possible without knowing whether its receptors exist on the membrane surface as independently diffusing monomers or as preassociated dimers or trimers, and there are no published data on this point. Our conclusions on this issue are therefore limited to the observation that receptor−ligand binding in this system is highly sensitive to avidity effects, in keeping with the multivalent nature of the complex, but the quantitative consequences of these effects for receptor engagement and signaling remain to be elucidated.

In mouse gene deletion experiments, the BAFF knockout and the BAFFR-deficient A/WySnJ mouse share many phenotypic traits such as a vastly reduced number of mature B cells and reduced serum immunoglobulin (*10*). Neither the BCMA nor the APRIL knockout had any obvious defect in B cell maturation or function and in fact had no overt phenotype. The observations that the BCMA knockout did not share any features of the BAFF knockout and the APRIL knockout showed none of the phenotypic traits seen with the BAFFR-deficient mouse are consistent with the notion that the interactions between these proteins that we have shown to be very weak *in vitro* also do not occur *in vivo*. Our results thus provide a molecular, mechanistic basis for understanding these genetic findings.

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REFERENCES

1. Moore, P. A., Belvedere, O., Orr, A., Pieri, K., LaFleur, D. W., Feng, P., Soppet, D., Charters, M., Gentz, R., Pamelee, D., Li, Y., Galperina, O., Giri, J., Roschke, V., Nardelli, B., Carrell, J., Sosnovtseva, S., Greenfield, W., Ruben, S. M., Olsen, H. S., Fikes, J., and Hilbert, D. M. (1999) BlyS: Member of the tumor necrosis factor family and B lymphocyte stimulator, *Science 285*, 260−263.
2. Schneider, P., MacKay, F., Steiner, V., Hofmann, K., Bodmer, J. L., Holler, N., Ambrose, C., Lawton, P., Bixler, S., Acha-Orbea, H., Valmori, D., Romero, P., Werner-Favre, C., Zubler, R. H., Browning, J. L., and Tschopp, J. (1999) BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth, *J. Exp. Med. 189*, 1747−1756.
3. Batten, M., Groom, J., Cachero, T. G., Qian, F., Schneider, P., Tschopp, J., Browning, J. L., and Mackay, F. (2000) BAFF mediates survival of peripheral immature B lymphocytes, *J. Exp. Med. 192*, 1453−1466.
4. Thompson, J. S., Schneider, P., Kalled, S. L., Wang, L., Lefevre, E. A., Cachero, T. G., MacKay, F., Bixler, S. A., Zafari, M., Liu, Z. Y., Woodcock, S. A., Qian, F., Batten, M., Madry, C., Richard, Y., Benjamin, C. D., Browning, J. L., Tsapis, A., Tschopp, J., and Ambrose, C. (2000) BAFF binds to the tumor necrosis factor receptor-like molecule B cell maturation antigen and is important for maintaining the peripheral B cell population, *J. Exp. Med. 192*, 129−135.
5. Wu, Y., Bressette, D., Carrell, J. A., Kaufman, T., Feng, P., Taylor, K., Gan, Y., Cho, Y. H., Garcia, A. D., Gollatz, E., Dimke, D., LaFleur, D., Migone, T. S., Nardelli, B., Wei, P., Ruben, S. M., Ullrich, S. J., Olsen, H. S., Kanakaraj, P., Moore, P. A., and Baker, K. P. (2000) Tumor necrosis factor (TNF) receptor superfamily member TACI is a high affinity receptor for TNF family members APRIL and BLyS, *J. Biol. Chem. 275*, 35478−35485.
6. Xia, X. Z., Treanor, J., Senaldi, G., Khare, S. D., Boone, T., Kelley, M., Theill, L. E., Colombero, A., Solovyev, I., Lee, F., McCabe, S., Elliott, R., Miner, K., Hawkins, N., Guo, J., Stolina, M., Yu, G., Wang, J., Delaney, J., Meng, S. Y., Boyle, W. J., and Hsu, H. (2000) TACI is a TRAF-interacting receptor for TALL-1, a tumor necrosis factor family member involved in B cell regulation, *J. Exp. Med. 192*, 137−143.
7. Yan, M., Marsters, S. A., Grewal, I. S., Wang, H., Ashkenazi, A., and Dixit, V. M. (2000) Identification of a receptor for BLyS demonstrates a crucial role in humoral immunity, *Nat. Immunol. 1*, 37−41.
8. Yu, G., Boone, T., Delaney, J., Hawkins, N., Kelley, M., Ramakrishnan, M., McCabe, S., Qiu, W. R., Kornuc, M., Xia, X. Z., Guo, J., Stolina, M., Boyle, W. J., Sarosi, I., Hsu, H., Senaldi, G., and Theill, L. E. (2000) APRIL and TALL-I and receptors BCMA and TACI: System for regulating humoral immunity, *Nat. Immunol. 1*, 252−256.
9. Groom, J., Kalled, S. L., Cutler, A. H., Olson, C., Woodcock, S. A., Schneider, P., Tschopp, J., Cachero, T. G., Batten, M., Wheway, J., Mauri, D., Cavill, D., Gordon, T. P., Mackay, C. R., and Mackay, F. (2002) Association of BAFF/BLyS overexpression and altered B cell differentiation with Sjogren's syndrome, *J. Clin. Invest. 109*, 59−68.
10. Schiemann, B., Gommerman, J. L., Vora, K., Cachero, T. G., Shulga-Morskaya, S., Dobles, M., Frew, E., and Scott, M. L. (2001) An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway, *Science 293*, 2111−2114.
11. Gross, J. A., Dillon, S. R., Mudri, S., Johnston, J., Littau, A., Roque, R., Rixon, M., Schou, O., Foley, K. P., Haugen, H., McMillen, S., Waggie, K., Schreckhise, R. W., Shoemaker, K., Vu, T., Moore, M., Grossman, A., and Clegg, C. H. (2001) TACI-Ig neutralizes molecules critical for B cell development and autoimmune disease. Impaired B cell maturation in mice lacking BLyS, *Immunity 5*, 289−302.
12. Hahne, M., Kataoka, T., Schroter, M., Hofmann, K., Irmler, M., Bodmer, J. L., Schneider, P., Bornand, T., Holler, N., and French, L. E. (1998) APRIL, a new ligand of the tumor necrosis factor family, stimulates tumor cell growth, *J. Exp. Med. 188*, 1185−1190.
13. Rennert, P., Schneider, P., Cachero, T. G., Thompson, J., Trabach, L., Hertig, S., Holler, N., Qian, F., Mullen, C., Strauch, K., Browning, J., Ambrose, C., and Tschopp, J. (2000) A soluble form of B cell maturation antigen, a receptor for the tumor necrosis factor family member APRIL, inhibits tumor cell growth, *J. Exp. Med. 192*, 1677−1683.
14. Varfolomeev, E., Kischkel, F., Martin, F., Seshasayee, D., Wang, H., Lawrence, D., Olsson, C., Tom, L., Erickson, S., French, D., Schow, P., Grewal, I. S., and Ashkenazi, A. (2004) APRIL-deficient mice have normal immune system development, *Mol. Cell. Biol. 24*, 997−1006.
15. Roschke, V., Sosnovtseva, S., Ward, C. D., Hong, J. S., Smith, R., Albert, V., Stohl, W., Baker, K. P., Ullrich, S., Nardelli, B., Hilbert, D. M., and Migone, T. S. (2002) BLyS and APRIL form biologically active heterotrimers that are expressed in patients with systemic immune-based rheumatic diseases, *J. Immunol. 169*, 4314−4321.
16. Laabi, Y., Gras, M. P., Carbonnel, F., Brouet, J. C., Berger, R., Larsen, C. J., Tsapis, A. (1992) A new gene, BCM, on chromosome 16 is fused to the interleukin 2 gene by a t(4;16)(q26;p13) translocation in a malignant T cell lymphoma, *EMBO J. 11*, 3897−3904.
17. Laabi, Y., Gras, M. P., Brouet, J. C., Berger, R., Larsen, C. J., and Tsapis, A. (1994) The BCMA gene, preferentially expressed during B lymphoid maturation, is bidirectionally transcribed, *Nucleic Acids Res. 22*, 1147−1154.
18. Gras, M. P., Laabi, Y., Linares-Cruz, G., Blondel, M. O., Rigaut, J. P., Brouet, J. C., Leca, G., Haguenauer-Tsapis, R., and Tsapis, A. (1995) BCMAp: An integral membrane protein in the Golgi apparatus of human mature B lymphocytes, *Int. Immunol. 7*, 1093−1106.
19. Xu, S., and Lam, K. P. (2001) B cell maturation protein, which binds the tumor necrosis factor family members BAFF and APRIL, is dispensable for humoral immune responses, *Mol. Cell. Biol. 21*, 4067−4074.
20. Thompson, J. S., Bixler, S. A., Qian, F., Vora, K., Scott, M. L., Cachero, T. G., Hession, C., Schneider, P., Sizing, I. D., Mullen, C., Strauch, K., Zafari, M., Benjamin, C. D., Tschopp, J., Browning, J. L., and Ambrose, C. (2001) BAFFR, a newly

identified TNF receptor that specifically interacts with BAFF, *Science 293*, 2108−2111.
21. Yan, M., Brady, J. R., Chan, B., Lee, W. P., Hsu, B., Harless, S., Cancro, M., Grewal, I. S., and Dixit, V. M. (2001) Identification of a novel receptor for B lymphocyte stimulator that is mutated in a mouse strain with severe B cell deficiency, *Curr. Biol. 11*, 1547−1552.
22. Miller, D. J., and Hayes, C. E. (1991) Phenotypic and genetic characterization of a unique B lymphocyte deficiency in strain A/WySnJ mice, *Eur. J. Immunol. 21*, 1123−1130.
23. von Bulow, G. U., and Bram, R. J. (1997) NF-AT activation induced by a CAML-interacting member of the tumor necrosis factor receptor superfamily, *Science 278*, 138−141.
24. von Bulow, G. U., van Deursen, J. M., and Bram, R. J. (2001) Regulation of the T-independent humoral response by TACI, *Immunity 14*, 573−582.
25. Seshasayee, D., Valdez, P., Yan, M., Dixit, V. M., Tumas, D., and Grewal, I. S. (2003) Loss of TACI causes fatal lymphoproliferation and autoimmunity, establishing TACI as an inhibitory BLyS receptor, *Immunity 18*, 279−288.
26. Banner, D. W., D'Arcy, A., Janes, W., Gentz, R., Schoenfeld, H. J., Broger, C., Loetscher, H., and Lesslauer, W. (1993) Crystal structure of the soluble human 55 kd TNF receptor-human TNF β complex: Implications for TNF receptor activation, *Cell 73*, 431−445.
27. Hymowitz, S. G., Christinger, H. W., Fuh, G., Ultsch, M., O'Connell, M., Kelley, R. F., Ashkenazi, A., and de Vos, A. M. (1999) Triggering cell death: The crystal structure of Apo2L/TRAIL in a complex with death receptor 5, *Mol. Cell. 4*, 563−571.
28. Mongkolsapaya, J., Grimes, J. M., Chen, N., Xu, X. N., Stuart, D. I., Jones, E. Y., and Screaton, G. R. (1999) Structure of the TRAIL−DR5 complex reveals mechanisms conferring specificity in apoptotic initiation, *Nat. Struct. Biol. 6*, 1048−1053.
29. Cha, S. S., Sung, B. J., Kim, Y. A., Song, Y. L., Kim, H. J., Kim, S., Lee, M. S., and Oh, B. H. (2000) Crystal structure of TRAIL−DR5 complex identifies a critical role of the unique frame insertion in conferring recognition specificity, *J. Biol. Chem. 275*, 31171−31177.
30. Cha, S. S., Kim, M. S., Choi, Y. H., Sung, B. J., Shin, N. K., Shin, H. C., Sung, Y. C., and Oh, B. H. (1999) 2.8 Å resolution crystal structure of human TRAIL, a cytokine with selective antitumor activity, *Immunity 11*, 253−261.
31. Singh, J., Garber, E., van Vlijmen, H., Karpusas, M., Hsu, Y. M., Zheng, Z., Naismith, J. H., and Thomas, D. (1998) The role of polar interactions in the molecular recognition of CD40L with its receptor CD40, *Protein Sci. 7*, 1124−1135.
32. Starling, G. C., Bajorath, J., Emswiler, J., Ledbetter, J. A., Aruffo, A., and Kiener, P. A. (1997) Identification of amino acid residues important for ligand binding to Fas, *J. Exp. Med. 185*, 1487−1492.
33. Starling, G. C., Kiener, P. A., Aruffo, A., and Bajorath, J. (1998) Analysis of the ligand binding site in Fas (CD95) by site-directed mutagenesis and comparison with TNFR and CD40, *Biochemistry 37*, 3723−3726.
34. Bajorath, J., Marken, J. S., Chalupny, N. J., Spoon, T. L., Siadak, A. W., Gordon, M., Noelle, R. J., Hollenbaugh, D., and Aruffo, A. (1995) Analysis of gp39/CD40 interactions using molecular models and site-directed mutagenesis, *Biochemistry 34*, 9884−9892.
35. Bodmer, J. L., Schneider, P., and Tschopp, J. (2002) The molecular architecture of the TNF superfamily, *Trends Biochem. Sci. 27*, 19−26.
36. Gordon, N. C., Pan, B., Hymowitz, S. G., Yin, J., Kelley, R. F., Cochran, A. G., Yan, M., Dixit, V. M., Fairbrother, W. J., and Starovasnik, M. A. (2003) BAFF/BLyS receptor 3 comprises a minimal TNF receptor-like module that encodes a highly focused ligand-binding site, *Biochemistry 42*, 5977−5983.
37. Liu, Y., Hong, X., Kappler, J., Jiang, L., Zhang, R., Xu, L., Pan, C. H., Martin, W. E., Murphy, R. C., Shu, H. B., Dai, S., and Zhang, G. (2003) Ligand−receptor binding revealed by the TNF family member TALL-1, *Nature 423*, 49−56.
38. Kim, H. M., Yu, K. S., Lee, M. E., Shin, D. R., Kim, Y. S., Paik, S. G., Yoo, O. J., Lee, H., and Lee, J. O. (2003) Crystal structure of the BAFF−BAFFR complex and its implications for receptor activation, *Nat. Struct. Biol. 10*, 342−348.
39. Kayagaki, N., Yan, M., Seshasayee, D., Wang, H., Lee, W., French, D. M., Grewal, I. S., Cochran, A. G., Gordon, N. C., Yin, J., Starovasnik, M. A., and Dixit, V. M. (2002) BAFF/BLyS receptor 3 binds the B cell survival factor BAFF ligand through a discrete surface loop and promotes processing of NF-κB2, *Immunity 17*, 515−524.
40. Wallweber, H. J., Compaan, D. M., Starovasnik, M. A., and Hymowitz, S. G. (2004) The crystal structure of a proliferation-inducing ligand, APRIL, *J. Mol. Biol. 343*, 283−290.
41. Hulme, E. C., and Birdsall, N. J. M. (1992) Strategy and tactics in receptor binding studies, in *Receptor−Ligand Interactions: A Practical Approach* (Hulme, E. C., Ed.) pp 63−176, Oxford University Press, New York.
42. *BIApplications Handbook* (1994) Chapter 6: Concentration measurement, pp 6-1−6-10, Pharmacia Biosensor AB.
43. Tribouley, C., Wallroth, M., Chan, V., Paliard, X., Fang, E., Lamson, G., Pot, D., Escobedo, J., and Williams, L. T. (1999) Characterization of a new member of the TNF family expressed on antigen presenting cells. *J. Biol. Chem. 380*, 1443−1447.
44. Shu, H. B., Hu, W. H., and Johnson, H. (1999) TALL-1 is a novel member of the TNF family that is down-regulated by mitogens, *J. Leukocyte Biol. 65*, 680−683.
45. Marsters, S. A., Yan, M., Pitti, R. M., Haas, P. E., Dixit, V. M., and Ashkenazi, A. (2000) Interaction of the TNF homologues BLyS and APRIL with the TNF receptor homologues BCMA and TACI, *Curr. Biol. 10*, 785−788.
46. Gross, J. A., Johnston, J., Mudri, S., Enselman, R., Dillon, S. R., Madden, K., Xu, W., Parrish-Novak, J., Foster, D., Lofton-Day, C., Moore, M., Littau, A., Grossman, A., Haugen, H., Foley, K., Blumberg, H., Harrison, K., Kindsvogel, W., and Clegg, C. H. (2000) TACI and BCMA are receptors for a TNF homologue implicated in B cell autoimmune disease, *Nature 404*, 995−999.
47. Pelletier, M., Thompson, J. S., Qian, F., Bixler, S. A., Gong, D., Cachero, T., Gilbride, K., Day, E., Zafari, M., Benjamin, C., Gorelik, L., Whitty, A., Kalled, S. L, Ambrose, C., and Hsu, Y. M. (2003) Comparison of soluble decoy IgG fusion proteins of BAFFR and BCMA as antagonists for BAFF, *J. Biol. Chem. 278*, 33127−33133.
48. Mackay, F., Woodcock, S. A., Lawton, P., Ambrose, C., Baetscher, M., Schneider, P., Tschopp, J., and Browning, J. L. (1999) Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations, *J. Exp. Med. 190*, 1697−1710.
49. Khare, S. D., Sarosi, I., Xia, X. Z, McCabe, S., Miner, K., Solovyev, I., Hawkins, N., Kelley, M., Chang, D., Van, G., Ross, L., Delaney, J., Wang, L., Lacey, D., Boyle, W. J., and Hsu, H. (2000) Severe B cell hyperplasia and autoimmune disease in TALL-1 transgenic mice, *Proc. Natl. Acad. Sci. U.S.A. 97*, 3370−3375.
50. Mackay, F., Schneider, P., Rennert, P., and Browning, J. (2003) BAFF AND APRIL: A tutorial on B cell survival, *Annu. Rev. Immunol. 21*, 231−264.
51. Patel, D. R., Wallweber, H. J., Yin, J., Shriver, S. K., Marsters, S. A., Gordon, N. C., Starovasnik, M. A., and Kelley, R. F. (2004) Engineering an APRIL-specific B cell maturation antigen, *J. Biol. Chem. 279*, 16727−16735.
52. Liu, Y., Xu, L., Opalka, N., Kappler, J., Shu, H. B., and Zhang, G. (2002) Crystal structure of sTALL-1 reveals a virus-like assembly of TNF family ligands, *Cell 108*, 383−394.
53. Dustin, M. L., Golan, D. E., Zhu, D. M., Miller, J. M., Meier, W., Davies, E. A., and van der Merwe, P. A. (1997) Low affinity interaction of human or rat T cell adhesion molecule CD2 with its ligand aligns adhering membranes to achieve high physiological affinity, *J Biol Chem. 272*, 30889−30898.

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