Engineering an APRIL-specific B Cell Maturation Antigen*

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B cell maturation antigen (BCMA) is a tumor necrosis factor receptor family member whose physiological role remains unclear. BCMA has been implicated as a receptor for both a proliferation-inducing ligand (APRIL) and B cell-activating factor (BAFF), tumor necrosis factor ligands that bind to multiple tumor necrosis factor receptors and have been reported to play a role in autoimmune disease and cancer. The results presented herein provide a dual perspective analysis of BCMA binding to both APRIL and BAFF. First, we characterized the binding affinity of monomeric BCMA for its ligands; BAFF binding affinity (IC50 = 8 ± 5 μM) is about 1000-fold reduced compared with the high affinity interaction of APRIL (IC50 = 11 ± 3 μM). Second, shotgun alanine scanning of BCMA was used to map critical residues for either APRIL or BAFF binding. In addition to a previously described “DXXLL” motif (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) Biochemistry 42, 5977–5983), the alanine scanning results predicted four amino acid positions in BCMA (Tyr13, Ile22, Gln25, and Arg27) that could impart ligand specificity. Substitution of Tyr13 was tolerated for BAFF binding but not APRIL binding. Arg27 was required for high affinity binding to APRIL, whereas substitutions of this residue had minimal effect on affinity for BAFF. Further phage display experiments suggested the single mutations of I22K, Q25D, and R27Y as providing the greatest difference in APRIL binding versus BAFF binding affinity. Incorporation of the Q25D and R27Y substitutions into BCMA produced a dual specificity variant, since it has comparable binding affinity for both APRIL and BAFF, IC50 = 350 and 700 nM, respectively. Binding of the I22K mutant of monomeric BCMA to BAFF was undetectable (IC50 > 100 μM), but affinity for binding to APRIL was similar to wild-type BCMA. Based on these results, a BCMA-Fc fusion with the single I22K mutation was produced that binds APRIL, IC50 = 12 nM, and has no measurable affinity for BAFF. These results suggest that APRIL is the preferred ligand for BCMA and show that specificity can be further modified through amino acid substitutions.

The tumor necrosis factor receptors (TNFRs)1 are a superfamily of transmembrane receptors involved in cell communication within the immune system. TNFR family members are structurally characterized by extracellular cysteine-rich domains (CRDs) that form ligand-binding motifs. Family members can be further classified based on intracellular domains that can either stimulate apoptosis through a death domain or cell proliferation through a TNF receptor-associated factor binding motif (1, 2). Downstream signaling for this subgroup of the TNFR superfamily activates the NF-κB intracellular pathway, often via TNF receptor-associated factors, ultimately resulting in cell proliferation (1–3). The corresponding TNF ligands share a common structural motif called the TNF homology domain, in the form of a β sheet jelly roll, through which the ligands primerize for receptor activation. TNF family members are expressed in a membrane-bound form but can undergo proteolysis to produce an active soluble trimer.

Generally, members of the TNFR superfamily found on B or T cells are type I transmembrane proteins that have several CRDs (1). There are, however, receptors in a subgroup that are expressed by B cells, are type III transmembrane proteins, and contain a reduced number of CRDs: B cell maturation antigen (BCMA), transmembrane activator and CAML interactor (TACI), and BlyS (BAFF) receptor 3 (BR3, also called BAFF-R) (4–8). The extracellular domain (ECD) of TACI contains two CRDs, the BCMA ECD comprises one CRD, and the ECD of BR3 contains only a partial CRD. Together with the receptor (Fn14) for the TWEAK ligand, BCMA and BR3 are the smallest members of the TNFR superfamily. Since TACI, BCMA, and BR3 lack an intracellular death domain, it is believed that these receptors are involved in cell survival, proliferation, and/or differentiation. Specifically, recent in vitro studies of BCMA presented evidence for the BCMA intracellular region interacting with TNF receptor-associated factors, leading toward downstream activation of NF-κB, ELK-1, c-Jun, and p38 pathways (3). BCMA homozygous knockout mice, however, show no distinct phenotype, suggesting a redundant or nonessential role for this receptor (9, 10). In contrast, recent in vivo studies with TACI reveal its critical role in B cell homeostasis and mice harboring a knockout of the TACI gene develop a fatal, lupus-like autoimmune disease (11). A mouse strain expressing a BR3 receptor having intact extracellular and transmembrane domains but a disrupted intracellular domain, and thus defective for downstream signaling, has a significantly reduced number of mature peripheral B cells (7). Thus, BR3 appears to be essential for B cell survival, TACI is important for modulating the B cell population, and the physiological role of BCMA is unclear.

The abbreviations used are: TNFR, tumor necrosis factor receptor; CRD, cysteine-rich domain; TNF, tumor necrosis factor; BCMA, B cell maturation antigen; TACI, transmembrane activator and CAML interactor; BAFF, B cell-activating factor; BR3, BlyS (BAFF) receptor 3; ECD, extracellular domain; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; NNS, (A/C/GT) (A/C/G/T) (G/C) codon.

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Ligand Specificity of Monovalent BCMA

The TNF family member BAFF is the only known ligand for BR3. BAFF-dependent B cell proliferation appears to require BR3; however, BAFF has also been reported to bind TACI and BCMA (6, 8, 12). APRIL, the TNF family member most closely related to BAFF, also binds TACI and BCMA (5, 7, 8). Despite cross-reactivity with receptors, the expression patterns of BAFF and APRIL are distinct; BAFF is expressed by macrophages, monocytes, neutrophils, dendritic cells, and radioreistant stromal cells, whereas APRIL is expressed by lymphoid cells and at elevated levels by some tumor cells (13–18). Tight regulation of BAFF levels appears to be critical for B cell homeostasis. BAFF knockout mice display significant reduction in the development and survival of follicular and marginal B cells, whereas mice expressing a BAFF transgene develop a lupus-like autoimmune syndrome (4, 9, 19, 20). Based on their roles in B cell-mediated immunity, these ligands (BAFF and APRIL) and their corresponding three receptors (BR3, BCMA, and TACI) provide likely targets for disease therapy. For example, studies by Kayagaki et al. (21) reveal attenuation of autoimmune lupus-like disease progression in mice with BR3-Fc treatment. In light of the receptor cross-reactivity, an APRIL-specific receptor would be a useful laboratory diagnostic tool or even a therapeutic agent; however, an APRIL-specific receptor has not yet been characterized.

Given that TNF ligands are trimeric and can bind three molecules of the corresponding receptor, there may be significant differences in apparent affinity between ligands binding to receptors in monovalent versus multivalent forms. Previous reports of binding affinities suggest that BAFF-BCMA has nanomolar affinity; however, these studies used a bivalent receptor-Fc fusion construct that could result in measured affinities that are enhanced by avidity (5, 6, 8). In this study, we characterized the APRIL and BAFF binding affinities of the extracellular domains of monomeric BCMA, BCMA-Fc, and BR3-Fc in order to determine the role that avidity could play in ligand-binding affinity. Furthermore, we have performed an alanine scan (22, 23) of the BCMA ECD in order to identify the residues determining specificity for binding to APRIL or BAFF. Finally, based on the alanine scan results, we produced a BCMA-Fc fusion with a single point mutation in the BCMA ECD, I22K, that maintains high affinity for APRIL but has no measurable binding to BAFF.

EXPERIMENTAL PROCEDURES

Reagents were obtained from the following sources:  o-phenylenediamine dihydrochloride (Sigma), Streptavidin peroxidase (Roche Applied Science), IgG-horseradish peroxidase (Jackson ImmunoResearch Laboratories), Protease Complete (Roche Applied Science), anti-M13-horseradish peroxidase (Roche Applied Science), sulfo-N-hydroxysuccinimide- biotin (Pierce).

Human BAFF was expressed and purified as previously described (24). Human BR3-Fc (25) was a kind gift from Y.-M. Hsu (Biogen). APRIL Expression and Purification—A PCR product coding for amino acids Lys104–Leu241 of murine APRIL was subcloned into the XbaI/NdeI sites of a modified pET-32a vector with a deleted S-Tag and enterokinase site to generate an N-terminal thioredoxin fusion protein. The pET-32a-APRIL (Lys104–Leu241) was transformed into Origami (DE3) competent cells (Novagen). Overnight cultures were diluted 1:100 and grown at room temperature in LB medium with 50 μg/ml carbenicillin to an A600 of 0.8 with vigorous shaking. Isopropyl-β-D-galactopyranoside was added to a final concentration of 1 mM for induction, and cultures were grown overnight at 25 °C. One liter of frozen cell pellet was resuspended in 100 mL of buffer A (50 mM Tris-HCl, pH 7.6, 300 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine) with 5 mM imidazole and plated on ice for 30 min. Cells were homogenized by passage through a microfluidizer and centrifuged at 15,000 rpm for 45 min. Supernatant was loaded onto a 3-mL nickel-nitrilotriacetic acid-agarose column (Qiagen), washed with 10 column volumes of Buffer A with 10 mM imidazole, and eluted with 10 column volumes of Buffer A with 300 mM imidazole. Fractions containing thioredoxin-APRIL fusion protein were pooled, concentrated, and purified by Superdex 200 size exclusion chromatography.

Baculovirus Expression and Purification of BCMA ECD—DNA coding for residues 4–53 of the human BCMA ECD was amplified by PCR and subcloned into the vector pET15b (Novagen) using the NdeI and Xhol restriction sites to introduce an N-terminal His tag and a thrombin cleavage sequence preceding the BCMA coding region. This His-tagged BCMA construct was subcloned into the baculovirus transfer vector pAcGFP1B (Clontech) using the BamHI and NotI restriction sites. The transfer vector was co-transfected with BaculoGold DNA into Sf9 cells, and recombinant virus was subsequently isolated and amplified to facilitate protein production. After 3 days of growth of virally infected Hs5 cells at 27 °C, His-tagged protein was purified from the infected medium by chromatography on Ni2+-nitrilotriacetic acid-agarose column (Qiagen), washed with 10 column volumes of Buffer A with 100 mM imidazole, and eluted with 10 column volumes of Buffer A with 300 mM imidazole. Fractions containing BCMA were pooled, concentrated, and purified by Superdex 25 column as a monomer. N-terminal sequencing and mass spectrometry were used to confirm the proper identity of the purified protein and the presence of both glycosylated and nonglycosylated species in the purified protein pool, respectively.

BCMA-Fc Expression and Purification—DNA encoding the ECD residues 5–51 of hBCMA was fused to hFc to form a fusion construct in the pRK vector and expressed in Chinese hamster ovary cells as previously described (5). Site-directed mutagenesis (Stratagene QuickChange method) was used to introduce the single mutant I22K in the BCMA-hFc fusion construct. E. coli BL21 (DE3) competent cells were transformed with the pRK-hBCMA-hFc plasmid for large scale production of plasmid DNA. Transient transfections of the plasmid in HEK 293 cells using FuGENE 6 (Roche Applied Science) produced secreted BCMA-Fc protein. The BCMA-Fc was purified from the HEK 293 growth medium by affinity chromatography using Protein A-Sepharose (Amersham Biosciences) resin.

Phage Display of BCMA—An initial vector for phage display of the BCMA extracellular domain was prepared by PCR subcloning of the fragment encoding residues 5–50 into the phagemid sTF-g3 (27). The resulting construct (BCMA1-g3) contained residues 5–50 fused at the C terminus to a tripeptide (G-S-A) linker and an amber stop codon following the C-terminal half of the M13 coat protein. The bacterial signal sequence stl1 was joined to the N terminus of BCMA with an inserted Ser residue comprising the P1′ cleavage site for the signal peptidase. Expression was driven by the alkaline phosphatase promoter. Phagemid BCMA2-g3 was prepared by using site-directed mutagenesis (28) to insert the peptide epitope (MADPNRFRGKDLGG) for an antibody (3C8:2F4; Genentech, Inc.) between the P1′ and P1 residues of the signal sequence cleavage site. BCMA2-g3 phagemid was used to prepare two “gunshot alanine” scanning libraries essentially as described previously (29). A shotgun alanine codon will code for the wild-type allele, alanine, or one of two additional substitutions in certain cases, due to codon degeneracy, at a given position. Each of these libraries, prepared separately, contains shotgun codons at unique positions. Library 1 has shotgun codons at positions 7, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, and 19; library 2 has shotgun codons at positions 22, 23, 25, 26, 27, 29, 30, 31, 32, 33, 34, 35, and 36. Each library contained at least 1 × 1013 phage/mL, allowing for complete representation of the theoretical diversity (>1010-fold excess) (library 1 codes 1.1 × 108 unique sequences, and library 2 codes 5.2 × 108 unique sequences).

Library Sorting and Analysis—Phage from each of the libraries described above were subjected to rounds of binding selection against either BAFF, APRIL, or anti-tag antibody (3C8:2F4; Genentech, Inc.) immobilized on 96-well Nunc Maxisorp immunoplates (30). Bovine serum albumin-coated wells were used to determine nonspecific background for binding. Phage eluted from each target screening selected using the same target in the previous round. Phage sorting was stopped, generally at round 2 or 3, when 100-fold enrichment was obtained. Enrichment was calculated from the ratio of the phage titer eluted from the target-coated wells to the phage titer from the bovine serum albumin-coated wells. For each library, the selection from each library and selection target were then grown in a 96-well format in 400 μL of 2T medium supplemented with carbenicillin and K07 helper phage; amplified phage were used for selection against the same target in the previous round. Phage ELISA assays (29) were performed to detect phage-displayed variants of BCMA ECD capable of binding BAFF, APRIL, or anti-tag antibody. All clones tested that were found to be positive for binding to BAFF, APRIL, or anti-tag antibody were sequenced with the LA PCR method previously (30). Sequences of acceptable quality were translated and aligned. For selection of binding to BAFF, 40 and 47 sequences were analyzed for libraries 1 and 2, respectively. For selection of binding to APRIL, 44 and 46 sequences were analyzed from libraries 1 and 2.
respectively. For the display selection, binding to anti-tag antibody, a maximum of 40 sequences were analyzed for each library. To quantify the effect of each mutation on ligand binding, normalized frequency ratios (F) for each amino acid position were calculated from a ratio of ligand selection to display efficiency selection, as described previously (31).

NNS Library Construction and Sorting—The BCM2-g3 phagemid was selected from a library having complete randomization in putative receptor-binding residues (see Discussion). Oligonucleotide-directed mutagenesis was performed as described (28) to generate point mutations in the target DNA. The mutagenesis was performed using a molar excess of biotin-sulfo-hydroxysuccinimide (Pierce) in PBS at 4°C for 3 h. After washing the NUNC plate coated with either APRIL or BAFF with PBS plus Tween 20, 100 μl of 1 M H3PO4, and the plate was incubated at room temperature. The plate was washed with PBS plus Tween 20 and incubated with 100 μl/ml of each receptor dilution for 1 h at room temperature. The plate was washed with PBS plus Tween 20, 100 μl/well of 1 M H3PO4, and the plate was incubated at 49 nM.

In a variation of the above ELISA, 3-fold serial dilutions of receptors were prepared in triplicate in PBS plus 0.05% Tween 20 with 7 μm biotinylated BCMA-Z (when APRIL was the target) or 0.5 μm biotinylated “mini-BR3,” BR3 residues 17–42 (when BAFF was the target). Mini-BR3 was prepared and biotinylated as described previously (24). For BCMA-Z biotinylation, 20 μg of purified BCMA-Z was incubated with a 3-fold molar excess of biotin-sulfo-N-hydroxysuccinimide (Pierce) in PBS at 25°C for 3 h. After washing the coated plate with PBS plus Tween 20, 100 μl/ml of each receptor dilution was transferred and incubated for 1 h at room temperature. The plate was washed with PBS plus Tween 20 and incubated with 100 μl/ml of 0.1 unit/ml Streptavidin peroxidase (Roche Applied Science) for 15 min. The peroxidase signal was developed and detected as described above. Measurements of Binding Constants for Receptors to Ligands—Surface plasmon resonance measurements on a BIAcore 3000 instrument (Pharmacia Biosensor) were used to measure binding affinities of receptors to immobilized APRIL and BAFF. The ligands were coupled to the sensor chip at a level of 40 resonance units using the amine coupling protocol supplied by the manufacturer. In all experiments, flow cell 1 was ethanolamine-blocked and used as the reference cell. Regeneration conditions that caused receptor dissociation, without disruption of the ligand trimers, could not be identified. Alternatively, in order to perform several binding measurements over the same flow cell, the ligand-receptor complex was allowed to dissociate by washing the flow cell with buffer at 30 μl/min for 900 s. Sensorgrams were recorded for receptor solutions ranging in concentration from 6.25 to 200 nM in 2-fold increments. Nonlinear regression analysis was used to simultaneously calculate kinetic constants and binding constants using software provided by the manufacturer.

RESULTS

BCMA Production—Previous reports on the interaction of BCMA with BAFF utilizing bivalent BCMA constructs (BCMA-Fc) have indicated a high affinity interaction with Kd values of about 1 nM (5, 8). More recently, studies with monovalent BCMA-Fc have indicated a much weaker affinity for BAFF (25). Here we have produced monomeric BCMA and measured affinities for both BAFF and APRIL. Monomeric BCMA was purified from baculovirus expression as well as from E. coli secretion as a fusion with the Z domain of Protein A. Although baculovirus expression provides a means to generate correctly folded and monomeric BCMA ECD, the Z domain fusion construct has the advantages of high level E. coli expression and detection through the Z-IgG interaction. BCMA-Z was readily purified from E. coli extracts by using a two-column procedure, and the purified protein eluted as a monomer from a gel filtration column (data not shown).

Measurement of Receptor-Ligand Binding Affinities—Binding affinity for monomeric human BCMA-Z to murine APRIL and human BAFF was measured by the competition ELISA described under “Experimental Procedures” (Fig. 1A). Although human BCMA and human BAFF were used in these experiments, murine APRIL was used, since the murine protein is better behaved than the human protein in vitro. Murine and human APRIL share >80% sequence identity, and the putative receptor-binding residues (see “Discussion”) are absolutely conserved. APRIL was expressed and purified as a thioredoxin fusion protein, and all binding experiments were done with the intact fusion protein, since removal of the thioredoxin by limited proteolysis resulted in reduced solubility. APRIL in solution was able to compete for binding to BCMA-Z with immobilized APRIL with an IC50 of 20 nM, whereas BAFF in solution competed for BCMA-Z binding with immobilized BAFF with an IC50 of >65 μM. Comparison of APRIL binding affinity for BCMA-Z and BCMA from baculovirus expression in a competition ELISA shows that the Z domain does not influence the measured affinity (Fig. 1B). This equivalent binding allowed the remaining BCMA binding measurements to be made using BCMA-Z. A competition ELISA measuring BCMA-Z binding to APRIL and BAFF using biotinylated BCMA-Z (for APRIL binding) or biotinylated mini-BR3 (24) (for BAFF binding) confirms that the affinity measurements of BCMA-Z binding to receptors (Fig. 1C) are consistent with the ligand competition data; IC50 values of BCMA-Z binding to APRIL (IC50 = 11 nM) are about 1000-fold higher in affinity than BCMA-Z binding to BAFF (IC50 = 8 μM for BAFF).

Since previously reported binding affinities of BCMA-BAFF interaction have been reported to be in the low nanomolar range (5, 8, 25), a comparison of various receptor constructs and ligands was done to examine the effects of avidity on receptor binding to APRIL and BAFF. Fig. 2 compares binding affinities of BCMA-Z, BCMA-Fc, and BR3-Fc to either BAFF (Fig. 2A) or APRIL (Fig. 2B). These data reveal that the monovalent BCMA-Z binds to BAFF (IC50 = 9 μM) with a 1000-fold reduced affinity compared with APRIL (IC50 = 7 nM), whereas BCMA-Fc, a bivalent construct, binds to both APRIL and BAFF with low nanomolar affinity (IC50 = 5 and 13 nM, respectively).
BR3-Fc binds BAFF, its predicted physiological ligand, with IC\textsubscript{50} = 9 nM for murine BR3-Fc and IC\textsubscript{50} = 7 nM for human BR3-Fc, but neither human nor murine BR3-Fc have measurable affinity for APRIL.

The ligand binding affinity of BCMA was also measured by surface plasmon resonance. APRIL and BAFF were selected for immobilization due to nonspecific interactions of APRIL with the sensor chip surface when used in the mobile phase. Table I summarizes the kinetics data for BCMA-Z and BCMA-Fc. BCMA-Z bound to immobilized APRIL with a $K_D$ value of 5.5 nM, whereas binding to BAFF was not detectable. BAFF binding by BCMA ECD produced via baculovirus expression was also undetectable. In contrast, BCMA-Fc bound to both BAFF ($K_D = 4.9$ nM) and APRIL ($K_D = 0.2$ nM) with high affinity, consistent with the competition ELISA result. BCMA-Fc binding to immobilized APRIL gave a faster off-rate with a slower on-rate than measured for the BCMA-Z-APRIL interaction, as expected for a bivalent molecule where avidity contributes to binding.

**Mutational Analysis of BCMA**—A shotgun alanine scan (29) of the single extracellular CRD of BCMA was used to determine the contribution of individual amino acid side chains to the binding of either APRIL or BAFF. In order to generate phage libraries that were completely represented by the phage pool,
the ECD was divided into two distinct libraries. In these libraries, wild-type codons were replaced by degenerate codons at amino acid positions 7–36 in the BCMA ECD (except cysteines and alamines), resulting in either the wild-type amino acid or alanine being expressed at the selected sites. For positions where the wild-type residue is Arg, Asn, Gln, His, Ile, Leu, Phe, and Tyr, the shotgun code allows for two additional amino acid substitutions, wild-type codons were replaced by degenerate codons at these positions followed by guest on July 26, 2018http://www.jbc.org/Downloaded from

The F value of an amino acid describes the effect of substitution on target binding relative to display efficiencies. F values of >1 represent deleterious mutations, whereas values of <1 represent favorable mutations. Due to the relatively small pool of enriched sequences analyzed, only F values representing a greater than 10-fold effect are considered significant. The Tyr13 position in BCMA tolerated some alanine, as well as aspartate and serine, substitution for BAFF-binding but was absolutely conserved for APRIL-binding. The F values for Phe14 indicate a significant contribution to BAFF binding but a more modest effect on affinity for APRIL. For both BAFF and APRIL binding, amino acids Asp15 and Leu17 are absolutely critical in that only wild-type residues were selected. A conservative substitution of Leu18 to Val was observed for both APRIL and BAFF selection; however, alanine was not observed at this position. Ala substitution of Ile22 was not tolerated for binding to BAFF but was compatible with APRIL binding. The Gln25 to Ala replacement appeared to have opposite effects on APRIL and BAFF binding, but the difference in F value was barely significant. In contrast, Ala substitution of Arg27 was strongly preferred for binding to BAFF and disfavored for APRIL binding. Other replacements in the C-terminal portion of BCMA had no effect on ligand binding.

Mutagenesis results for residues Ile22, Gln25, and Arg27 suggest these positions as likely candidates for providing ligand specificity, since Ala substitution had opposite effects on BAFF and APRIL binding. Positions Leu18, Ile22, Gln25, and Arg27 were selected for further phage optimization studies by incorporation of NNS degenerate codons at these positions followed by selection for ligand binding. This new phage library was subjected to three rounds of sorting against either BAFF or APRIL and compared with the display target antibody. A large F value for an amino acid at a given position indicates that that amino acid is a favorable substitution for binding to the target ligand. As shown in Table II, the substitutions that result in the maximum difference in F (23, 32) between APRIL binding and BAFF binding are I22K, Q25D, and R27Y. The I22K substitution produced a >12-fold preference for APRIL binding over BAFF binding; Q25D and R27Y resulted in >13- and 9.5-fold preference for APRIL binding relative to APRIL binding, respectively.

In order to confirm the effects on ligand binding indicated by the phage display results, point mutants were produced as BCMA-Z fusion proteins, purified, and assayed for BAFF and APRIL binding by competitive displacement ELISA (Table III). These data show that Tyr13 is critical for BCMA binding to APRIL, since Ala, Ser, or Phe substitutions of this residue all increased the IC50 by at least 400-fold. The substitution of Tyr13 with Ala, Ser, or Phe, produced only small changes in affinity for BAFF. Consistent with the phage display results,
the single amino acid substitution of I22K in BCMA-Z caused a greater than 10-fold reduction in affinity for BAFF but only about a 3-fold penalty for APRIL binding. The single substitution of Q25D in BCMA-Z did not significantly improve binding to BAFF relative to APRIL. The double mutant Q25D/R27Y reduced APRIL binding 40-fold, compared with wild type. The double mutant Q25D/R27Y produced a receptor that could bind both APRIL and BAFF with nearly the same affinity. These substitutions gave nonadditive contributions to binding, since the effect measured for the double mutant was greater than the sum of effects measured for the single mutants. For example, the Q25D substitution resulted in decreased affinity for BAFF, but when combined with R27Y it gave an increased affinity.

Surface plasmon resonance was used to independently measure the binding constants of the mutant BCMA-Z proteins for APRIL (Table IV). Consistent with ELISA IC50 measurements, BCMA-Z wild-type, I22K, and Q25D all bound APRIL in the low nanomolar range. R27Y BCMA-Z binding to APRIL showed a 1000-fold reduction in affinity. The decreased affinity of this mutant results primarily from a decrease in the on rate of binding, which leads to poor fitting to a 1:1 binding model and probably underestimates the actual $K_D$. The surface plasmon resonance data for BCMA-Z Q25D/R27Y binding to APRIL was
Ligand Specificity of Monovalent BCMA

APRIL (IC50 values shown are for BCMA-Z wild type (mean ± S.D. of four data sets) or BCMA-Z point mutants competing with biotinylated BCMA-Z for binding to immobilized APRIL or competition with biotinylated BR3 for binding to BAFF.

| BCMA-Z | IC50 values (µM) | BAFF | APRIL |
|--------|-----------------|------|------|
| Wild type | 8 ± 5 | 11 ± 3 |
| Y13A | 12 | 5100 |
| Y13S | 6 | 8400 |
| Y13F | 3 | 5700 |
| I22K | >100 | 38 |
| Q25D | 36 | 32 |
| R27Y | 4 | 400 |
| Q25D/R27Y | 0.7 | 350 |

TABLE IV

BCMA-Z binding to immobilized APRIL by surface plasmon resonance

| BCMA-Z | kₐ | kₚ | K_D |
|--------|----|----|-----|
| Wild type | 8.5 | 4.7 | 5.5 |
| I22K | 11.0 | 6.9 | 6.3 |
| Q25D | 7.9 | 5.9 | 7.5 |
| R27Y | 0.1 | 25 | 6500 |

not well described by a 1:1 binding model such that the rate and equilibrium constants could not be calculated.

Measurement of BCMA-Fc Wild Type and I22K Binding to Ligands—Since the single point mutation I22K resulted in a BCMA-Z protein that maintained high affinity for APRIL and no measurable binding to BAFF, this mutation was introduced into BCMA-Fc protein to determine whether the affinity and specificity would be present in a bivalent format. The competition ELISA method, as described under “Experimental Procedures,” was used to measure binding of BCMA-Fc wild type and I22K fusion proteins to APRIL or BAFF using biotinylated BCMA-Z for APRIL binding or biotinylated BR3 (24) for BAFF binding (Fig. 4). These data reveal that the single point mutation, I22K, in BCMA resulted in a bivalent protein that binds APRIL (IC50 = 12 nM) with similar affinity to the wild type BCMA-Fc (IC50 = 9 nM) but has no measurable affinity to BAFF (IC50 > 10 µM).

DISCUSSION

Monovalent BCMA binds APRIL with high affinity. In contrast, the affinity of BCMA for BAFF is 1000-fold weaker than for APRIL. Since the BCMA produced in E. coli binds APRIL with high affinity and is equivalent to material produced by expression in insect cells, the weak affinity for BAFF cannot be explained by a misfolding of BCMA-Z. A recent report also described the weak affinity of the monovalent BCMA-BAFF interaction (25); however, ours is the first report of a high affinity interaction between monovalent BCMA and APRIL. Consistent with earlier studies (5, 8, 25), the bivalent protein (BCMA-Fc) binds BAFF with an apparent high affinity. An avidity component originating from a bivalent receptor interacting with a ligand having three binding sites can enhance the apparent affinity. Thus, it is likely that BAFF may bind with high affinity to cells expressing BCMA only if the receptors are preorganized on the cell surface. Although it has been proposed that FAS and TNFR1 can form homo-oligomers on the cell surface in the absence of ligand (37), BCMA appears to lack the PLAD domain necessary for this association. Under normal physiological conditions, BCMA may operate only as a receptor for APRIL and not for BAFF. This conclusion is consistent with recent findings showing that the effects of BAFF on the development of B cells are independent of BCMA (9).

Similar to BR3, a DXL motif is presented at the tip of a type I β-turn in BCMA (21, 36). Shotgun alanine scanning of BCMA confirms that the DXL motif is critical for APRIL binding as well as BAFF binding (Fig. 5A). These results suggest that this portion of BCMA is bound in a cavity on APRIL that is similar to the pocket on BAFF for binding BCMA and BR3 (38–40). In the structures determined for the BR3-BAFF or BCMA-BAFF (Fig. 5B) complexes, the leucine residue of the DXL motif (Leu28 in BR3, Leu17 in BCMA) interacts with BAFF residues Ala207, Leu111, Ile233, and Pro264, with Gly209 forming the bottom of the pocket. The Asp residue of the DXL motif (Asp26 in BR3, Asp15 in BCMA) makes a salt bridge with BAFF residue Arg265, and the confirmation of the Asp may be stabilized through a hydrogen bond with BAFF residue Tyr206. High resolution structures for APRIL by itself or in complex with BCMA are not available; however, APRIL is expected to have a similar binding pocket for the leucine side chain, since, as shown in Fig. 5B, the key residues are conserved (Gly209, Ile233, and Pro264) or conservatively substituted (A207T, L211V), Arg265 is conserved in APRIL, thus allowing formation of a salt bridge with Asp15.

Residues outside of the DXL motif would appear to confer the specificity of BCMA for APRIL and BR3 for BAFF. For example, mutation of Tyr15 has little effect on BAFF-binding (Table III), but even the subtle substitution of Tyr15 for phenylalanine results in a remarkable 520-fold decrease in APRIL binding affinity. The BAFF-BCMA crystal structure (36) shows that the hydroxyl group of Tyr15 has the potential to form hydrogen bonds with Asp15 and Arg27 on BCMA and/or Tyr206 on BAFF (Fig. 5). Our results indicate that none of these potential hydrogen bonds are important for BAFF binding. In contrast, the hydroxyl group is clearly essential for high affinity APRIL binding. Interestingly, the residue corresponding to Tyr206 in APRIL is a phenylalanine, suggesting that the importance of Tyr15 probably results from an intramolecular hydrogen bond within BCMA. Given the key role of Asp15 in the interface, the importance of Tyr15 to APRIL binding may be due to an indirect effect of influencing the position of Asp15; in BAFF, such a role could be accomplished by Tyr206 on the ligand.
Arg27 of BCMA is also important for specifying APRIL versus BAFF-binding. Again, mutation of this position has little effect on BAFF binding but disrupts APRIL binding significantly (Tables III and IV). In the BAFF-BCMA crystal structure, Arg27 forms a salt bridge with Glu266, yet the R27Y mutant indicates that this salt bridge is not required for BAFF association. This conclusion is consistent with the observation of a high affinity BAFF-BR3 interaction, since BR3 has a leucine (Leu38) in the position analogous to Arg27. Since APRIL has Glu266 replaced with Ala, it is unclear why Arg27 of BCMA is required for high affinity binding to APRIL. Further understanding of this effect will require determination of the BCMA-APRIL complex structure. In addition to the contributions from Leu38 interactions, the specificity of BR3 for BAFF over APRIL could be explained by the substitution of a cysteine residue (Cys24), which makes a disulfide bond with Cys25 for the residue equivalent to Tyr27. Indeed, the C24Y mutation in BR3 increases affinity for APRIL (36), and hydrophobic residues at position 38 in BR3 are important for BAFF binding (24).

In addition to BCMA residues Tyr13 and Arg27, our results from phage display experiments suggest that Ile22 and Gln25 are important for determining ligand specificity. Ile22 makes a hydrophobic contact with BAFF, and thus the I22K substitution results in a weaker affinity for BAFF. Interestingly, wild-type murine BCMA has Lys at position 22 and would likely have a weak affinity for mBAFF. The contact residues for Ile22 on BAFF, Tyr206, and Leu240, are replaced with Phe and Arg in APRIL. Given the positive charge on APRIL residue Arg240, it is surprising that the I22K substitution has no effect on affinity for APRIL. The side chain of Gln25 is not in contact with BAFF, and thus the Q25D single mutation has no significant effect on APRIL or BAFF binding. However, Q25D did increase affinity for BAFF when combined with R27Y such that a dual specificity BCMA variant was obtained. Given that Arg27 and Gln25 point in opposite directions, the origin of this nonadditive effect is unclear.

Both BAFF and APRIL have been implicated in autoimmune diseases and carcinomas. APRIL expression has been shown to be up-regulated in many tumors including colon and prostate cancers (13, 41, 42). The BAFF-specific antagonist BR3-Fc (21),
Ligand Specificity of Monovalent BCMA

16735

together with studies on BAFF knockout mice (9), has been used to demonstrate the essential role of BAFF in B cell development. The role of APRIL in B cell homeostasis is unclear, especially since APRIL knockout mice display normal B cell levels (43). Soluble BCMA-Fc has been used to suggest a role in vivo.

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