Stoichiometry of Heteromeric BAFF and APRIL Cytokines Dictates Their Receptor Binding and Signaling Properties[*]

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Background: The B cell survival factors B cell activation factor (BAFF) and a proliferation-inducing ligand (APRIL) can heteromerize.
Results: BAFF-APRIL$_2$ and APRIL-BAFF$_2$ heteromers have distinct receptor-binding specificities and activities.
Conclusion: BAFF-APRIL$_2$ resembles APRIL, and APRIL-BAFF$_2$ resembles BAFF but poorly activates the BAFF receptor.
Significance: Heteromers should be taken into account when evaluating the physiology or pharmacological inhibition of BAFF and APRIL.

The closely related TNF family ligands B cell activation factor (BAFF) and a proliferation-inducing ligand (APRIL) serve in the generation and maintenance of mature B-lymphocytes. Both BAFF and APRIL assemble as homotrimers that bind and activate several receptors that they partially share. However, heteromers of BAFF and APRIL that occur in patients with autoimmune diseases are incompletely characterized. The N and C termini of adjacent BAFF or APRIL monomers are spatially close and can be linked to create single-chain homo- or heteroligands of defined stoichiometry. Similar to APRIL, heteromers consisting of one BAFF and two APRILs (BAA) bind to the receptors B cell maturation antigen (BCMA), transmembrane activator and CAML interactor (TACI) but not to the BAFF receptor (BAFFR). Heteromers consisting of one APRIL and two BAFF (ABB) bind to TACI and BCMA and weakly to BAFFR in accordance with the analysis of the receptor interaction sites in the crystallographic structure of ABB. Receptor binding correlated with activity in reporter cell line assays specific for BAFFR, TACI, or BCMA. Single-chain BAFF (BBB) and to a lesser extent single-chain ABB, but not APRIL or single-chain BAA, rescued BAFFR-dependent B cell maturation in BAFF-deficient mice. In conclusion, BAFF-APRIL heteromers of different stoichiometries have distinct receptor-binding properties and activities. Based on the observation that heteromers are less active than BAFF, we speculate that their physiological role might be to down-regulate BAFF activity.

BAFF,[3] also known as BLyS (B lymphocyte stimulator), is a TNF family ligand primarily expressed by myeloid cells and radiation-resistant stromal cells of secondary lymphoid organs. Together with its close relative APRIL, BAFF acts on peripheral B cells at various stages of differentiation through three receptors as follows: BAFFR that responds to BAFF only, and TACI and BCMA that bind to both BAFF and APRIL (1). BAFFR controls the survival and metabolic fitness of peripheral B cells, with the exception of memory cells, plasma cells, and B1 B cells (2–4). BAFFR is highly expressed in naive B cells but decreases and finally completely disappears during activation and differentiation of B cells to plasma cells. In contrast, TACI is transiently expressed in B cells upon activation, for example in response to Toll-like receptor ligands (5). Its expression is particularly high in marginal zone B cells, but, as for BAFFR, it is not maintained in plasma cells. The role of TACI is complex. It is required for efficient antibody production in response to type II T-independent antigens, but at the same time it exerts a negative role on B cells. Indeed, TACI-deficient mice display higher numbers of B cells than their wild type counterparts (6, 7). In marginal zone B cells, simultaneous engagement of TACI and Toll-like receptors induces expression of both Fas and FasL to help contract this innate-like type of response by apoptotic cell death at a later stage (8). Finally, BCMA is expressed late during B cell differentiation and is probably the prime receptor mediating the action of BAFF and APRIL on plasma cells (2, 9). The

blockade of BAFF with a monoclonal antibody has proven to be moderately effective in the autoimmune disease systemic lupus erythematosus, and several other reagents blocking either BAFF alone or BAFF and APRIL are presently in clinical development (10).

BAFF exists in membrane-bound (11) or -soluble (12) forms, and the latter can crystallize as 3- or 60-mers (13, 14). Although APRIL is mainly released in a soluble form (15), it can bind to proteoglycans and thus create niches for antibody-secreting cells (16, 17). Heteromers of BAFF and APRIL are produced in autoimmune diseases and are sometimes also detected in healthy subjects (18, 19). Because of the intrinsic difficulty of producing and purifying BAFF-APRIL heteromers, these ligands have remained incompletely characterized. Here, we produced single-chain heteromers of BAFF and APRIL with defined compositions; we crystallized one of them and characterized their receptor-binding profile, their activities, and their susceptibility to BAFF or BAFF and APRIL inhibitors.

Experimental Procedures

Animals—C57BL/6 WT and BAFF-KO were as described (20). Mice were handled according to guidelines and under the authorization of the Swiss Federal Food Safety and Veterinary Office (authorization 1370.6 to P. S.).

Plasmids and Recombinant Proteins—Expression plasmids used in this study were constructed using standard molecular biology techniques. Proteins they code for are described in Table 1. Atacicept was provided by Merck, KGaA. Belimumab (registered trade name Benlysta) was purchased from the Pharmacy of Lausanne University Hospital. Other recombinant proteins were produced essentially as described (21, 22).

Transfections—HEK 293T cells were grown in DMEM, 10% fetal calf serum (FCS) and transfected by the calcium phosphate or polyethyleneimide methods. Cells were grown for 7 days in serum-free Opti-MEM medium (Invitrogen) for the production of single-chain lymphotoxins or for 48 h in complete medium for surface expression of receptors:TRAILR3 fusion proteins.

CHO cells (Sigma) grown in DMEM/F-12 (1:1) supplemented with 2% FCS were transfected with polyethylenimide or Polyfect (Qiagen) and selected 48 h later in medium containing 500 μg/ml G418 sulfate (Calbiochem). Cells were cloned by limiting dilution or by two or three rounds of FACS sorting when constructs of interest were linked to green fluorescent protein expression via an internal ribosome entry site sequence. The best producing clones were selected.

Protein Production and Purification—Conditioned supernatants were affinity-purified on anti-FLAG M2-agarose (Sigma) or protein A-Sepharose (GE Healthcare) for FLAG- and Fc-tagged ligands, respectively, essentially as described (21). Single-chain ligands were subsequently fractionated by size exclusion chromatography on a Superdex 200 column (GE Healthcare) equilibrated in PBS. Protein concentrations were inferred from the extinction coefficient calculated from the theoretical amino acid sequence. Untagged single-chain heteromers were obtained by secretion from HEK 293 cells with N-terminal His$_6$-FLAG tags, affinity chromatography on nickel-nitrilotriacetic acid, tag cleavage with tobacco etch virus protease, and size exclusion chromatography on a Superdex 200

column. The final purified proteins were prepared in 20 mM Hepes/NaOH, pH 7.5, 150 mM NaCl and were concentrated on 30-kDa ultrafiltration devices (Vivascience).

SDS-PAGE and Western Blot—SDS-PAGE and Western blot were performed according to standard procedures. Proteins were revealed directly with horseradish peroxidase-coupled anti-human IgG antibody or in two steps with anti-FLAG M2 at 1 μg/ml, anti-hBAFF Buffy-2 rat IgM at 1 μg/ml, or anti-hAPRIL Aprily-2 mouse IgG1 at 1 μg/ml, followed by appropriate horseradish peroxidase-coupled secondary reagents. Coomassie blue staining was performed with a semidry iD Stain System (Eurogentec).

Receptor-Ligand Interaction ELISA—The direct binding of FLAG-tagged ligands to immobilized receptor-Fcs, either adsorbed to or captured via anti-human antibodies in 96-well immunoplates, was revealed with biotinylated anti-FLAG M2 antibody (Sigma) and horseradish-coupled streptavidin as described previously in detail (22). For the competition ELISA, titrated amounts of untagged ligands were added to immobilized receptor-Fcs for 30 min and followed, without intermediate washing step, by an empirically determined fixed and nonsaturating concentration of FLAG-tagged ligand sufficient to generate ~80% of the maximal signal in the absence of competitor (22).

Cytotoxic Assays with Receptor:Fas Reporter Cell Lines—Jurkat BCMA:Fas (cl13) and Jurkat JOM2 TACI:Fas (cl112.3) have been described (20, 23). Similarly, Jurkat JOM2 BAFFR:Fas cell lines (cl21) were generated as described in detail elsewhere by retrovirus-mediated transduction of a BAFFR:Fas chimeric receptor, puromycin selection, and screening of clones for their selective ability to respond to Fc-BAFF by apoptotic cell death (22). Reporter cells were incubated for 16 h with the indicated concentrations of ligands, after which time cell viability was measured by the phenazine methosulfate/3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium assay essentially as described (22). FLAG-tagged ligands were used in the presence of 1 μg/ml anti-FLAG M2 antibody.

Crystallography—Crystals of the single-chain APRIL-BAFF-BAFF heterotrimer (*His$_6$-FLAG-TEV-GS*-hAPRIL (aa 111–250, T126A)-GGGGS-hBAFF(140–285)-GGGGS-hBAFF(140–285), with cleaved tags) were obtained by mixing 0.5 μl of protein solution (6 mg/ml in 20 mM Hepes/NaOH, pH 7.5, 150 mM NaCl) with 0.5 μl of reservoir solution (0.1 M Tris/HCl, pH 8.75, 14% PEG6000 (w/v), and 1 M LiCl) using the hanging drop vapor diffusion method at 293 K. Before flash-freezing in liquid nitrogen, crystals were cryo-protected by reservoir solution supplemented with 10% (v/v) 2,3-butanediol.

Diffraction data were collected at 100 K at beamline X06SA (Swiss Light Source, Villigen, Switzerland). Data were processed to 2.43 Å resolution using the programs XDS and XSCALE (24). The crystals belong to space group C 2 2 21 containing two heterotrimers per asymmetric unit. Initial phases were obtained by molecular replacement using Phaser and the published structures of APRIL (Protein Data Bank code 1Q5X) and BAFF (Protein Data Bank code 1KD7) as search models (25). Multiple rounds of manual model building in COOT and bulk solvent correction, positional, B-factor, and

TABLE 1

Plasmids used in this study

aa is amino acid. HA signal = MAIIYLILLFTAVRG Ig signal = MNFGFSLIFLVLVLKG CamLinker = PQPQPKPQPKPEPEGS PreSci = LEVLFQGP TEV = ENLYFQ.

Plasmid	Designation	Protein encoded	Vector
ps193	FLAG-hTNF	HA signal-FLAG-GPGQVQLQ-hTNF (aa 85–263)	PCR3
ps420	hTNFR1-Fc	hTNFR1 (aa 1–217)-VD-hIgG1 (aa 245–470)	PCR3
ps515	EGFP	Enhanced green fluorescent protein	PCR3
ps645	FLAG-hLTa	HA signal-FLAG-GPGQVQLQVD-hLTa (aa 43–205)	PCR3
ps739	hBCMA-Fc	Ig signal-EVKLVPRGS-hBCMA (aa 2–54)-VD-hIgG1 (aa 245–470)	PCR3
ps897	hTACI-GPI	HA signal-LD-hTACI (aa 2–160)-VD-hTRAILR3 (aa 157–269)	PCR3
ps1196	Fc-hBAFF	HA signal-LD-hIgG1 (aa 245–470)-RS-CamLinker-PreSci-GSLQ-hBAFF (aa 137–285)	PCR3
ps1377	pMSCV-puro	Modified pMSCV-puro (<zcoclontech>Clontech) with HindIII-BglII-EcoRI-NotI-XhoI-HpaI-ApaI cloning sites	ps1377
ps1406	Fc-hLTb	HA signal-LD-hIgG1 (aa 245–470)-RS-CamLinker-PreSci-GSLQ-hLTb (aa 66–244)	PCR3
ps1467	hBCMA-GPI	Ig signal-VQCEVKLVPRGS-hBCMA (aa 2–54)-VD-hTRAILR3 (aa 157–269)	PCR3
ps1589	hBAFFR-GPI	HA signal-LE-hBAFFR (aa 2–71)-EFGSVD-hTRAILR3 (aa 157–269)	PCR3
ps1799	mLTbR-Fc	mLTbR (aa 1–220)-VD-PreSci-hIgG1 (aa 245–470)	PCR3
ps2297	mBAFFR-Fc	HA signal-LD-mBAFFR (aa 2–70)-VD-hIgG1 (aa 245–470)	PCR3
ps2308	hBAFFR:Fas	HA signal-LE-hBAFFR (aa 2–71)-EFGSVD-hFas (aa 169–355)	ps1377
ps2309	hBCMA:Fas	Ig signal-VQCEVKLVPRGS-hBCMA (aa 2–54)-VD-hFas (aa 169–335)	ps1377
ps2455	hTACI:Fas	HA signal-L-hTACI (aa 1–118)-VD-hFas (aa 169–335)	ps1377
ps2642	FLAG-hLT-ααα	HA signal-FLAG-GPGQVQLQVD-hLTa (aa 58–205)-GGGGS-hLTa (aa 58–205)-GGGGS-hLTa (aa 58–205)	PCR3
ps2643	FLAG-hLT-ααβ	HA signal-FLAG-GPGQVQLQVD-hLTa (aa 58–205)-GGGGS-hLTa (aa 58–205)-GGGGS-hLTb (aa 83–244)	PCR3
ps2644	FLAG-hLT-ββα	HA signal-FLAG-GPGQVQLQ-hLTb (aa 83–244)-GGGGS-hLTb (aa 83–244)-GGGGS-hLTa (aa 58–205)	PCR3
ps2645	FLAG-hLT-βββ	HA signal-FLAG-GPGQVQLQ-hLTb (aa 83–244)-GGGGS-hLTb (aa 83–244)-GGGGS-hLTb (aa 83–244)	PCR3
ps2680	Fc-BBB	HA signal-LD-hIgG1 (aa 245–470)-RS-CamLinker-LQ-hBAFF (aa 140–285)-GGGGS-hBAFF (aa 140–285)-GGGGS-hBAFF (aa 140–285)	PCR3
ps2826	Fc-hAPRIL	HA signal-LD-hIgG1 (aa 245–470)-RS-CamLinker-GSLQ-hAPRIL (aa 115–250)	PCR3
ps2876	Fc-AAA	HA signal-LD-hIgG1 (aa 245–470)-RS-CamLinker-LQ-hAPRIL (aa 112–250)-GGGGS-hAPRIL (aa 112–250)-GGGGS-hAPRIL (aa 112–250)	PCR3
ps2877	Fc-BAA	HA signal-LD-hIgG1 (aa 245–470)-RS-CamLinker-LQ-hBAFF (aa 140–285)-GGGGS-hAPRIL (aa 112–250)-GGGGS-hAPRIL (aa 112–250)	PCR3
ps2879	FLAG-AAA	HA signal-FLAG-GPGQVQLQ-hAPRIL (aa 112–250)-GGGGS-hAPRIL (aa 112–250)-GGGGS-hAPRIL (aa 112–250) IRES GFP	PCR3
ps2883	Fc-ABB	HA signal-LD-hIgG1 (aa 245–470)-RS-CamLinker-LQ-hAPRIL (aa 112–250)-GGGGS-hBAFF (aa 140–285)-GGGGS-hBAFF (aa 140–285)	PCR3
ps2886	FLAG-ABB	HA signal-FLAG-GPGQVQLQ-hAPRIL (aa 112–250)-GGGGS-hBAFF (aa 140–285)-GGGGS-hBAFF (aa 140–285) IRES GFP	PCR3
ps2889	FLAG-BAA	HA signal-FLAG-GPGQVQLQ-hBAFF (aa 140–285)-GGGGS-hAPRIL (aa 112–250)-GGGGS-hAPRIL (aa 112–250) IRES GFP	PCR3
ps2890	FLAG-BBB	HA signal-FLAG-GPGQVQLQ-hBAFF (aa 140–285)-GGGGS-hBAFF (aa 140–285)-GGGGS-hBAFF (aa 140–285) IRES GFP	PCR3
ps3413	hTACI-Fc	Modified Ig signal (METDTLLLWVLLLWVPGVHG)-hTACI (aa 31–110) hIgG1 (aa 245–470; L258E, A353S, P354S)	PCR3
pcDNA3.1_AAB	AAB	Ig signal-His6-FLAG-TEV-GS-hAPRIL (aa 111–250; T126A)-GGGGS-hAPRIL (aa 111–250; T126A)-GGGGS-hBAFF (aa 140–285)	pcDNA3.1
pcDNA3.1_ABA	ABA	Ig signal-His6-FLAG-TEV-GS-hAPRIL (aa 111–250; T126A)-GGGGS-hBAFF (aa 140–285)-GGGGS-hAPRIL (aa 111–250; T126A)	pcDNA3.1
pcDNA3.1_BAA	BAA	Ig signal-His6-FLAG-TEV-GS-hBAFF (aa 140–285)-GGGGS-hAPRIL (aa 111–250; T126A)-GGGGS-hAPRIL (aa 111–250; T126A)	pcDNA3.1
pcDNA3.1_BBA	BBA	Ig signal-His6-FLAG-TEV-GS-hBAFF (aa 140–285)-GGGGS-hBAFF (aa 140–285)-GGGGS-hAPRIL (aa 111–250; T126A)	pcDNA3.1
pcDNA3.1_BAB	BAB	Ig signal-His6-FLAG-TEV-GS-hBAFF (aa 140–285)-GGGGS-hAPRIL (aa 111–250; T126A)-GGGGS-hBAFF (aa 140–285)	pcDNA3.1
pcDNA3.1_ABB	ABB	Ig signal-His6-FLAG-TEV-GS-hAPRIL (aa 111–250; T126A)-GGGGS-hBAFF (aa 140–285)-GGGGS-hBAFF (aa 140–285)	pcDNA3.1

TABLE 2
Crystallographic data and model refinement statistics

Data collection

X-ray source	X06SA / SLS[1]
Wavelength (Å)	0.99998
Space group	C222$_1$
Unit-Cell parameters [Å, °]	a=57.04; b=117.86; c=295.52; $\alpha = 90.0$ $\beta = 90.0$ $\gamma = 90.0$
Resolution [Å] [2]	2.43 (2.68-2.43)
Number of measurements [2]	134837 (34739)
Number of unique reflections [2]	36901 (9358)
Completeness [%] [2]	96.7 (97.9)
Multiplicity	3.7 (3.7)
R_{sym} [%][2,5]	7.9 (50.5)
R_{meas} [%][2,4]	9.2 (58.7)
Mean(I)/sd [2,5]	13.46 (3.06)

Refinement

Resolution [Å]	147.76-2.43
Number of reflections (test)	35790 (1111)
R_{work}/R_{free}	17.9 / 23.4
Rmsd bond length [Å][6]	0.01
Rmsd bond angle [°][6]	1.22
Number of atoms	
Protein atoms	6644
Ligand	8
Water	339
Ramachandran[7]	
Favored [%]	96.0
Outliers [%]	0.5

1: Swiss Light Source (SLS, Villigen, Switzerland).
2: values in parenthesis refer to the highest resolution bin.

$$3: \quad Rsym = \frac{\sum_h \sum_i^{n_h} \left| \hat{I}_h - I_{h,i} \right|}{\sum_h \sum_i^{n_h} I_{h,i}} \quad \text{with} \quad \hat{I}_h = \frac{1}{n_h} \sum_i^{n_h} I_{h,i}$$

where $I_{h,i}$ is the intensity value of the *i*th measurement of *h*.

$$4: \quad Rmeas = \frac{\sum_h \sqrt{\frac{n_h}{n_h - 1}} \sum_i^{n_h} \left| \hat{I}_h - I_{h,i} \right|}{\sum_h \sum_i^{n_h} I_{h,i}} \quad \text{with} \quad \hat{I}_h = \frac{1}{n_h} \sum_i^{n_h} I_{h,i}$$

where $I_{h,i}$ is the intensity value of the *i*th measurement of *h*.
5: calculated from independent reflections.
6: Root mean square deviations from geometric target values.
7: Calculated from MolProbity.

TLS refinement using REFMAC and BUSTER yielded the final model (26 –29). Data collection and model statistics are shown in Table 2. The atomic coordinates and structure factors have been deposited in the Protein Data Bank, Protein Data Bank code 4ZCH. Images were generated with the PyMOL Molecular Graphics System, Schrödinger, LLC.

Administration of Proteins to BAFF-deficient and Wild Type Animals—Fc-BBB, Fc-ABB, Fc-BAA, Fc-APRIL, and PBS were administered intraperitoneally to BAFF-KO mice at 1 mg/kg on days 0, 4, 7, 11, and 14. Untreated wild type mice were used as controls. Mice were sacrificed at day 18, and spleens and lymph nodes (inguinal, axillary, and brachial) were collected, homog-enized, incubated in ACK lysis buffer (150 mM NH$_4$Cl, 10 mM KHCO$_3$, 10 μM Na$_2$-EDTA) for 5 min on ice to lyse red blood cells, washed in PBS 2% FCS, and filtered on a nylon mesh.

FACS Analyses—293T cells co-transfected with EGFP and receptor-GPI expression plasmids were stained with titrated amounts of FLAG- or Fc-tagged ligands, whose binding was revealed with appropriate phycoerythrin-coupled secondary re-agents, as described (22, 30). Cells were analyzed using FACScan or Accuri 6 flow cytometers (BD Biosciences) and FlowJo software (TreeStar, Ashland, OR).

Splenocytes or lymph node cells were incubated with anti-CD16/32 (Fc-block, clone 93) and stained with a mix of anti-CD19-PE.Cy7 (clone eBio1D3, 1:200), anti-CD93-biot (mAb493, 1:100, a kind gift of Antonius Rolink, University of Basel, Switzerland), and anti-CD3-APC (clone 17A2, 1:100) (all from eBiosciences) for 20 min on ice followed by phycoerythrin Cy5.5-coupled streptavidin (1:100) and analysis with a FACS Canto (BD Biosciences). Data were analyzed with the FlowJo software.

Results

Production of Functional Single-chain Heteromers of LTα and -β with Defined Stoichiometry—TNF family members share a common structural fold, the TNF homology domain, that trimerizes to form receptor-binding sites at the interface of ligand subunits. In the structure of TNF and of several other TNF family ligands, the N- and C-terminal regions of mono-meric ligand subunits are only 5–11 Å away from each other (14, 31, 32), making it possible to link three individual mono-mers within a trimer by replacing the Stop codon of the first and second monomers by short linker peptides (sequence GGGGS) to produce single-chain ligands (Fig. 1*A*). TNF is known to remain active as a single-chain ligand (33). We first validated the single-chain ligand approach using lymphotoxin-α and -β (LTα and LTβ). LTα and LTβ form both homo- and hetero-trimers of physiologic relevance that differ in their receptor-binding profile: LTα binds to TNFR1 and LT$\alpha_1\beta_2$ binds to LTβR (34). Moreover, single-chain LT$\alpha_1\beta_2$ remains active (35).

Single-chain FLAG-tagged constructs expressing LTα and LTβ in various combinations ($\alpha\alpha\alpha$, $\alpha\alpha\beta$, $\beta\beta\alpha$, and $\beta\beta\beta$, theo-retical molecular weight of 51.5, 52.4, 53.0, and 53.9) were all successfully expressed and secreted by 293T cells (Fig. 1*B*). Conventional FLAG-TNF, FLAG-LTα, or FLAG-LTα co-ex-pressed with Fc-LTβ were used as controls. As expected, FLAG-TNF and FLAG-LTα bound TNFR1, but not LTβR, in an ELISA-based assay, and the same was true for single-chain LTα-LTα-LTα (Fig. 1*C*). LT-$\beta\beta\alpha$, the *bona fide* ligand for LTβR, and single-chain LTβ-LTβ-LTβ bound to LTβR but not to TNFR1, whereas single-chain LTα-LTα-LTβ showed an intermediate specificity with some binding to both TNFR1 and LTβR (Fig. 1*C*). Taken together, these results indicate that both LTα and LTβ retain the ability to interact with cognate recep-tors when modified at their C termini and expressed as single-chain ligands. The observation that LT-$\beta\beta\alpha$ did not bind to TNFR1 strongly suggests that, as expected, the stoichiometry is fixed in single-chain ligands. Indeed, if α subunits from differ-ent single-chain heteromers had the capacity to assemble into
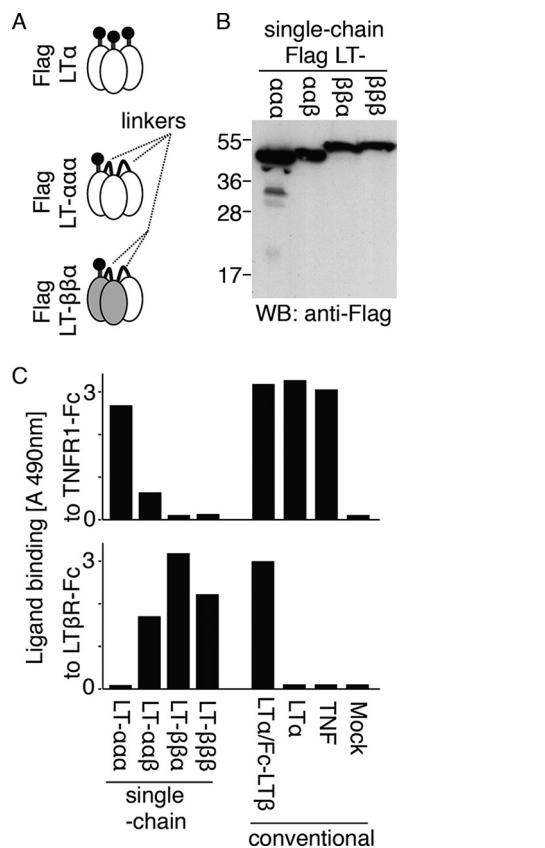
FIGURE 1. **Receptor-binding specificity of lymphotoxin-$\alpha\beta$ heteromers of defined stoichiometries.** *A,* schematic representation of "conventional" (FLAG LTα) and single-chain ligands (FLAG single-chain LTα-LTα-LTα, FLAG LT-$\beta\beta\alpha$) in which individual monomers are covalently linked by a flexible linker of five amino acid residues. *B,* anti-FLAG Western blot (*WB*) of the indicated single-chain FLAG-tagged lymphotoxins. *C,* binding of the indicated FLAG-tagged conventional or single-chain ligands to immobilized TNFR1-Fc (*upper panel*) or LTβR-Fc (*lower panel*), as measured in an ELISA-based assay. LTα/Fc-LTβ is FLAG-LTα co-expressed with Fc-LTβ.

pure LTα trimers, binding to TNFR1 should have been detected, which was not the case.

Expression and Purification of Single-chain BAFF, APRIL, and BAFF-APRIL Heteromers—Affinity-purified FLAG- and Fc-tagged single-chain ligands produced in CHO cells migrated by SDS-PAGE with sizes of about 60 and 100 kDa, respectively, corresponding roughly to the predicted molecular masses of the glycosylated proteins (Fig. 2*A* and Table 3). A Western blot analysis confirmed the presence of both BAFF and APRIL in the single-chain heteromers and of either BAFF or APRIL in the homomers (Fig. 2*A*). Despite several expression attempts, single-chain APRIL was only obtained in low yield, especially when Fc-tagged.

FLAG-tagged ligands were eluted by size exclusion chromatography with apparent molecular masses 1.5–1.7-fold higher than the expected ones (Table 3). We find it unlikely that these molecules assemble as dimers and favor the hypothesis that the proteins have either nonglobular shapes and/or that the contribution of *N*-linked glycans to the hydrodynamic volume is higher than anticipated.

Fc-tagged single-chain ligands migrated by size exclusion chromatography with apparent sizes 2.8 to 5 times that of the monomeric single chain (Table 3), although in theory, given the
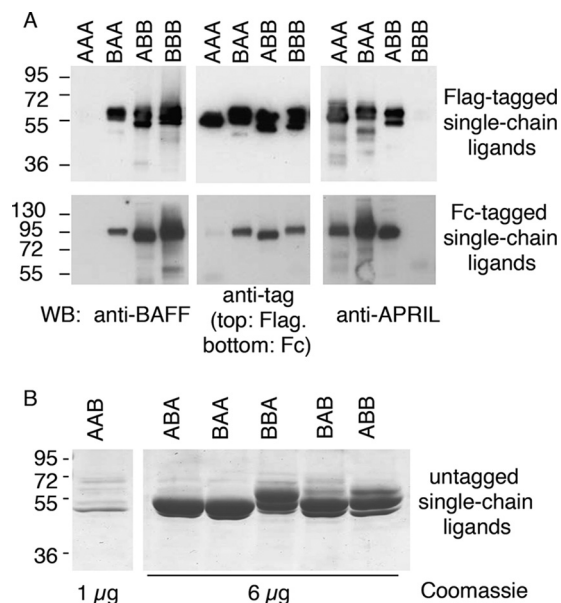

FIGURE 2. **SDS-PAGE analysis of single-chain BAFF-APRIL heteromers.** *A,* Western blot analysis with 200 ng/lane of FLAG-tagged or 100 ng/lane of Fc-tagged single-chain BAFF-APRIL heteromers (except for Fc-AAA, ~20 ng) revealed as indicated with either anti-BAFF Buffy-2, anti-FLAG M2, anti-APRIL Aprily-2 monoclonal antibodies, or with an anti-Fc antibody. *B,* Coomassie Blue staining of 6 μg/lane (except for AAB, 1 μg) of the indicated untagged single-chain BAFF-APRIL heteromers. *WB,* Western blot.

dimeric nature of the Fc, it should have been only twice the size. Sizes of nonglobular proteins are overestimated by size exclusion chromatography, and we hypothesize that the presence of rod-like linkers (PQPQPKPQPKPEPEGS) between the Fc portion and the single-chain ligands might have contributed to the observed migration. Fc-tagged ABB and BBB were bigger by size exclusion chromatography than Fc-tagged BAA and AAA, and they might represent structures composed of four single chains (*i.e.* containing two dimeric Fc). When present, aggregates eluting in the void volume had been analyzed separately and share the same receptor-binding properties as nonaggregated ligands (data not shown).

Single-chain heteromers of BAFF and APRIL constructed in six possible combinations (AAB, ABA, BAA, ABB, BAB, and BBA) were also produced as untagged proteins in HEK 293 cells (Fig. 2*B*). In these constructs, one *N*-glycosylation site of APRIL was mutated (T126A). These proteins migrated by SDS-PAGE and size exclusion chromatography with sizes compatible with the theoretical ones (Fig. 2*B* and Table 3).

Taken together, these results indicate that single-chain homomers and heteromers of BAFF and APRIL can be produced but for single-chain APRIL only in low yield. All experiments were performed with size-fractionated ligands to exclude contributions of high molecular weight aggregates.

Crystal Structure of Single-chain APRIL-BAFF-BAFF Heteromer—The structure of untagged single-chain ABB determined by crystallography at a resolution of 2.43 Å revealed the expected trimeric arrangement between the APRIL and the two BAFF subunits (Fig. 3, *A* and *B*). Interfaces between BAFF and APRIL involve residues that are mostly identical or conserved between both ligands, with conserved residues present at the core of the interaction surface, explaining why heteromeriza-

TABLE 3
Sizes of single-chain constructs of BAFF and APRIL

Constructs[a]	Theoretical no. of N-linked[b]	Theoretical approximate mass (+N-linked)[c]	Observed approximate mass, WB + DTT	Observed approximate mass, S200	Ratio, S200/WB
		kDa	*kDa*	*kDa*	
FLAG-AAA	6	64.3	58	85	1.5
FLAG-BAA	5	62.5	60	90	1.5
FLAG-ABB	4	60.9	59	100	1.7
FLAG-BBB	3	59.3	58	95	1.6
Fc-AAA	7	92.7	98	270	2.8
Fc-BAA	6	91	98	330	3.4
Fc-ABB	5	89.4	93	500	5.4
Fc-BBB	4	88	100	500	5.0
BAA, ABA, AAB	3	56.1	51[d]	40	0.8
ABB, ABA, BBA	3	56.8	54[e]	45	0.8

[a] Tagged constructs were produced in CHO cells, and untagged constructs were produced in HEK 293 cells.
[b] Number of consensus N-glycosylation sites (NX(S/T)) is shown.
[c] We assume that all consensus sites are glycosylated and counted 2.5 kDa per N-linked glycan.
[d] 51- and 54-kDa bands were observed and are probably N-glycosylation species.
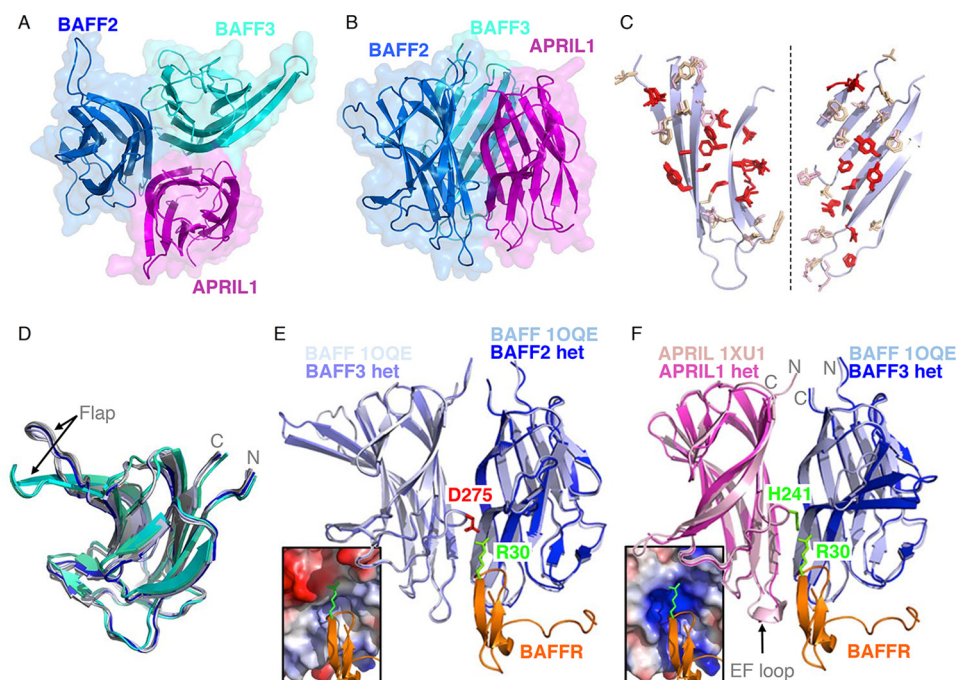[e] 51-, 54-, and 57-kDa bands were observed and are probably N-glycosylation species.

FIGURE 3. Structural features of an APRIL-BAFF-BAFF heteromer. *A,* top view of a single-chain APRIL-BAFF-BAFF heteromer, with APRIL in *magenta,* and both BAFFs (BAFF2 and BAFF3) in *blue* and *cyan. Ribbon* drawing of the structure, in a semi-transparent space-filling representation. *B,* side view of the APRIL-BAFF-BAFF heteromer with the same color code as in *A. C,* residues that are involved in protomer interactions and that are conserved in the five interfaces examined (BAFF-BAFF in 1OQE, APRIL-APRIL in 1XU1, BAFF3-BAFF2, APRIL1-BAFF3, and BAFF2-APRIL1 in ABB) are shown in *red.* Other residues of BAFF (*wheat*) or APRIL (*light pink*) that participate in protomer-protomer interactions are also shown. Portions of the relevant β-sheets of BAFF are shown in *pale blue* (β-sheets of APRIL, not shown, are essentially superimposable). The two protomers were rotated by about 90° and −90°, respectively, so that both sides of the interaction surface face the reader. *D,* ribbon representation of superimposed monomers of BAFF taken from structures of BAFF 3-mers (shades of *light blue,* 1KD7 and 1KXG), BAFF 60-mers (shades of *gray,* 1ODQ, 1OQE, and 4V46), and ABB heteromer (second BAFF domain, *dark blue*; third BAFF domain, shades of *cyan,* as found in the two heteromers of the crystallization unit). The position of the DE loop (Flap) and of the N and C termini are indicated. *E,* two BAFF protomers (*light shades* of *blue*) and one BAFFR molecule (*orange*) taken from the crystal structure of BAFF 60-mer in complex with BAFFR (1OQE) were superimposed with the two adjacent BAFF domains (in *dark shades* of *blue*) of single-chain ABB. The side chains of Arg-30 of BAFFR and Asp-275 of BAFF, which are both determinants of BAFFR specificity for BAFF and not APRIL, are shown in *green* and *red,* respectively. The electrostatic potential of the relevant portion of the BAFF-BAFF interface in the heteromer is shown in the *inset,* from *red* (negative) to *blue* (positive). *F,* relevant ligand subunits of BAFF (1OQE), APRIL (1XU1), and single-chain ABB were superimposed. APRIL and BAFF subunits of the ABB heteromer are shown in *dark shades* of *magenta* and *blue,* respectively. One BAFF subunit (*light blue*) and one BAFFR (*orange*) of the BAFF-BAFFR complex (1OQE) are also shown, together with a relevant subunit of mouse APRIL (*pink*) of the APRIL-TACI complex (1XU1). N and C termini are indicated. Side chains of Arg-30 of BAFFR and His-241 of APRIL are shown in *green.* The electrostatic potential of the APRIL-BAFF interface is shown in the *inset.*

tion can take place (Fig. 3*C*). Subunits of BAFF monomers adopt a folding very similar to that of BAFF previously crystallized as 3- or 60-mers (r.m.s.d. of 0.47 to 0.54 Å) (Fig. 3*D*) (13, 14, 36–38), and the same was true for the APRIL subunit that resembled previously crystallized mouse APRIL (r.m.s.d. of 1.56 or 0.76 Å when excluding loop EF that is different in both structures) (Fig. 3*F*) (32). In particular, the N and C termini of

BAFF and APRIL in the heteromer superimposed with those of conventional BAFF and APRIL, indicating that GGGGS linkers between BAFF and APRIL subunits have no noticeable impact on the structure (Fig. 3, *D–F*). Except for a single glycine residue at the C terminus of the central BAFF subunit, linkers were flexible and unresolved in the structure. It is noteworthy that BAFF subunits at the C termini of both ABBs in the asymmetric

unit of the crystal displayed a striking structural difference in the "flap" region, *i.e.* the loop between β-sheets D and E (r.m.s.d. of 2.25 Å between BAFF2 and BAFF3, but only 0.4 Å when excluding the DE loop). In these BAFF subunits, the flap adopts a different but well structured conformation in which the DE β-pleated sheet is longer, and the loop is reduced in size (Fig. 3D).

The receptor binding regions of the heteromer were examined for their predicted ability to accommodate BAFFR. Most TNF family receptors have elongated extracellular domains that contact ligands at the interface of two adjacent ligand protomers (39, 40). In contrast, BAFFR mainly contacts a single BAFF protomer (36, 37). Arg-30 of BAFFR, a known determinant of its specificity for BAFF and not APRIL (41), is also contacted by Asp-275 of the adjacent BAFF protomer (Fig. 3E). APRIL has a histidine at the corresponding position (His-241), and compared with the BAFF-BAFF interface, the APRIL-BAFF interface more generally lacks the negatively charged microenvironment that accommodates Arg-30 of BAFFR (Fig. 3F). Thus, ABB may present only one high affinity site for BAFFR instead of three in BBB, which would result in a decreased avidity for BAFFR.

In summary the crystal structure of single-chain ABB indicates the following: (i) linkers do not interfere with folding and assembly of BAFF and APRIL subunits; (ii) the structure of human and mouse APRIL monomers are very similar; (iii) the flap of the C-terminal BAFF subunit of ABB adopts a structure that has never been observed before; and (iv) ABB is predicted to bind BAFFR with a lower avidity than BAFF.

Receptor Binding Specificity of Single-chain BAFF, APRIL, and BAFF-APRIL Heteromers—The binding of FLAG-tagged single-chain ligands to immobilized BAFFR-Fc, BCMA-Fc, and TACI-Fc was characterized in an ELISA-based assay. FLAG-BBB bound BAFFR, BCMA, and TACI but not the irrelevant TNFR1, and FLAG-AAA only bound BCMA and TACI, as observed previously for conventional ligands. For heteromers, FLAG-BAA behaved like AAA. FLAG-ABB bound all three receptors, but its binding to BAFFR, and to a lesser extent TACI, was clearly less efficient than that of BBB (Fig. 4A). In line with these results, untagged heteromers were unable to compete for FLAG-BAFF binding to BAFFR, indicating that the binding affinity of ABB (and BAB and BBA) for BAFFR is lower than that of BAFF (Fig. 4B). In contrast, all heteromers could compete with FLAG-BAFF binding to BCMA, indicating that BAFF and BAFF-APRIL heteromers have similar affinities for BCMA (Fig. 4B).

The receptor-binding specificity of FLAG-tagged heteromers was also tested in a FACS-based assay, in which GPI-anchored receptors were expressed in 293T cells (Fig. 4C) (30). Binding results were virtually identical to those of the ELISA-based assay (Fig. 4D). Taken together, these data indicate that BAA is close to APRIL in terms of receptor binding and that ABB in addition displays some binding to BAFFR, but not to the same level as BAFF.

Activity of Single-chain BAFF, APRIL, and BAFF-APRIL Heteromers on Reporter Cells—FLAG-tagged single-chain ligands were tested for their ability to induce death in apoptosis-sensitive Jurkat T cells expressing the extracellular domains

of BAFFR, BCMA, or TACI fused to the transmembrane domain and intracellular tail of the death-inducing receptor Fas. Thus, successful binding and oligomerization of the receptor:Fas fusion proteins by BAFF or APRIL induces death of target cells through the surrogate Fas pathway. As triggering of the Fas pathway is intimately linked to the multimerization state of ligands (42), FLAG-tagged single-chain ligands were incubated in the presence of a cross-linking anti-FLAG antibody. All ligands killed TACI:Fas cells and BCMA:Fas cells, although AAA and to a lesser extent BAA were more active than ABB and BBB on BCMA:Fas cells. On BAFFR:Fas reporter cells, BBB was about 300-fold more efficient than ABB, in line with the poor binding of ABB to BAFFR (Figs. 4 and 5A). As expected from binding data, BAA and AAA have virtually no activity on BAFFR:Fas cells (Fig. 5A). Similar results were obtained for Fc-tagged single-chain ligands, except that differences between ligands were less marked than with FLAG-tagged ligands when assayed on TACI:Fas reporter cells (Fig. 5B). Whether this was due to an increased avidity of dimeric Fc ligands for TACI or to differences in protein preparation or assay conditions is not known. Finally, untagged single-chain ligands displayed activities mostly in line with those of the corresponding FLAG-tagged proteins, and there were no major differences between heterotrimers in which subunits of identical composition were present in a different order (Fig. 5C). Taken together, these results indicate that the activity of single-chain ligands on receptor:Fas cells reflects quite precisely their receptor binding ability and that the order in which BAFF and APRIL subunits are fused within a single-chain heteromer appears to be indifferent.

BAFF and APRIL Heteromers Are Differentially Inhibited by BAFF and APRIL Antagonist Drugs—Belimumab is an anti-BAFF antibody approved for the treatment of systemic lupus erythematosus, and atacicept is a TACI:Fc fusion protein currently in a phase II clinical trial for the same disease. The ability of these agents to inhibit BAFF-APRIL heteromers of defined stoichiometry was investigated. At a few-fold mass excess over the ligand, atacicept blocked not only BAFF and APRIL but also both types of heteromers (BAA and ABB) (Fig. 6). In contrast, belimumab efficiently inhibited Fc-BAFF and Fc-BBB but had very little to no activity on Fc-BBA, Fc-BAA, Fc-APRIL, and Fc-AAA (Fig. 6). Thus, the specificity of belimumab is limited to trimeric BAFF, whereas atacicept neutralizes BAFF and APRIL trimers and all heteromeric configurations thereof.

Activity of Single-chain BAFF and BAFF-APRIL Heteromers on Primary B Cells in Vivo—Although assays performed in the first part of this study are well defined from a molecular point of view and informative regarding receptor binding specificity of BAFF-APRIL heteromers, they do not address the capacity of heteromers to activate full-length receptors expressed at endogenous levels in a natural environment. To determine in particular whether the weak binding of ABB to BAFFR is physiologically relevant, Fc-tagged single-chain ligands were administered to BAFF-KO mice. Indeed recombinant BAFF can rescue a functionally mature B cell compartment in these mice, at least to some extent (20, 43). In BAFF-KO mice, CD93-positive immature B cells are normal, but there are only few CD93-negative mature B cells in the spleen and few mature B cells in
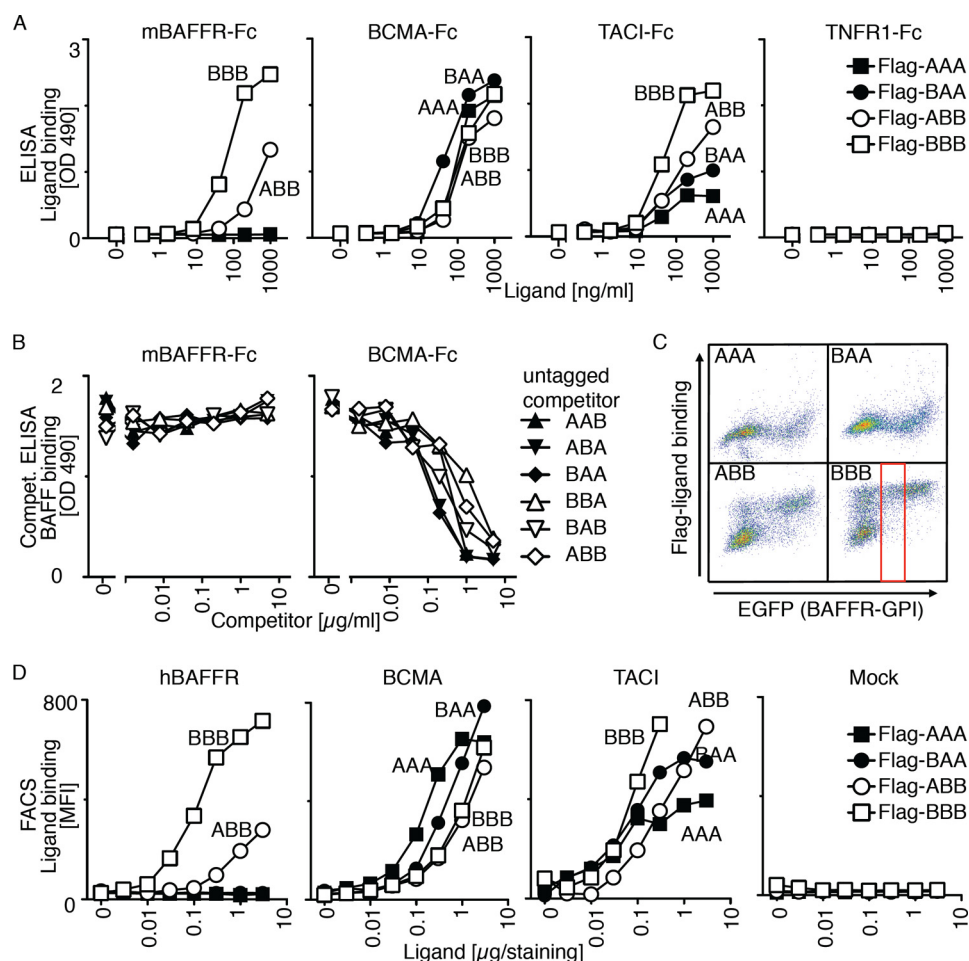
FIGURE 4. **Receptor binding specificity of BAFF-APRIL heteromers.** *A,* binding of titrated amount of FLAG-tagged single-chain BAFF-APRIL heteromers to the indicated immobilized receptors-Fc was monitored in an ELISA-based assay. *B,* binding of a constant, nonsaturating amount of FLAG-BAFF to mBAFFR-Fc or BCMA-Fc was monitored in an ELISA-based assay. Before the addition of FLAG-BAFF, coated receptor Fcs were preincubated with titrated amounts of the indicated untagged single-chain BAFF-APRIL heteromers that served as competitors for FLAG-BAFF. *C,* 293T cells were co-transfected with plasmids coding for the following: (*a*) the extracellular domain of hBAFFR fused to the C-terminal GPI addition signal of TRAILR3, and (*b*) EGFP as a fluorescent tracer. These cells were stained with the indicated FLAG-tagged single-chain BAFF-APRIL heteromers (at 1 μg/staining = 40 μg/ml). FACS scattergrams show the binding of ligands (*y* axis) as a function of EGFP expression (which itself is proportional to BAFFR-GPI). Both axes show fluorescence intensities (from 10^0 to 10^4 arbitrary units) on a log scale. The gate shows the population of cells with intermediate EGFP expression that was selected to quantify mean fluorescence intensity in *D*. *D,* mean fluorescence intensity (*MFI*) of the binding of titrated amounts of the indicated single-chain FLAG-tagged heteromers to 293T cells expressing hBAFFR-GPI, BCMA-GPI, TACI-GPI or no transfected receptor (*Mock*). Experiment was performed as described in *C*. Experiments shown in *A–D* are representative of at least two with similar results.

lymph nodes (Fig. 7). As observed previously for Fc-BAFF, administration of Fc-BBB at 1.5 mg/kg for 2 weeks to BAFF-KO mice rescued both mature splenic and lymph node B cells (Fig. 7) (43). Fc-ABB was less efficient than Fc-BBB in this respect, but nevertheless it significantly increased the number of mature splenic B cells. There was also a trend for increased lymph node B cells in response to Fc-ABB, which did not reach statistical significance (Fig. 7). Finally, as anticipated, Fc-BAA and Fc-APRIL that do not bind to BAFFR had no effect on mature B cell populations (Fig. 7). Taken together, these results indicate that BAFF-rich heteromers, but not APRIL-rich ones, have the potential to functionally stimulate BAFFR in primary B cells *in vivo* and to allow B cell maturation.

Discussion

The trimeric nature of both BAFF and APRIL makes the production and characterization of heteromers thereof a challenging issue, first to separate heteromers from homotrimers and

then to resolve heteromers of different compositions. Although this is possible and has already been done for lymphotoxins (44) and for the APRIL$_2$BAFF$_1$ heteromer (18), it is difficult to exclude that exchanges of protomers at equilibrium might restore a proportion of homotrimers in a purified heteromer. In this regard, the strategy of expressing TNF family ligands as single-chain proteins, which was initially developed to specifically introduce mutations in one but not all subunits of TNF (45), has two undeniable advantages as follows: the generation of heteromers with defined stoichiometry, and a guarantee against exchanges of subunits. The size of FLAG-tagged and untagged heteromers and the structure of ABB undoubtedly confirmed that these constructs really formed heteromers. Interestingly, interfaces between adjacent BAFF-BAFF, APRIL-APRIL, BAFF-APRIL, or APRIL-BAFF subunits are virtually identical in the central region of the interaction, and they often involve similar residues in the peripheral part of the interaction (Fig. 3C). A potential concern with single-chain ligands is the
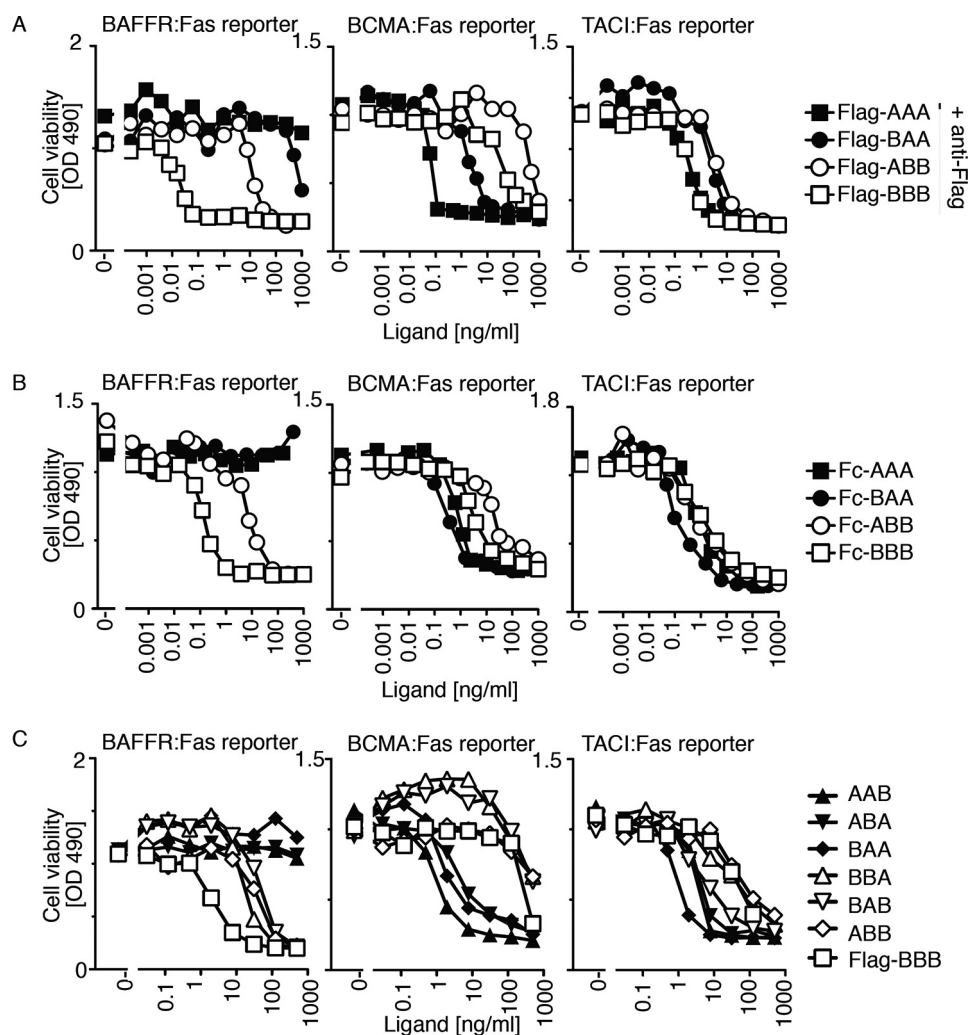
FIGURE 5. **Activity of BAFF-APRIL heteromers on BAFFR-, BCMA-, and TACI-specific reporter cell lines.** Clones of Jurkat cells stably transduced with the chimeric receptors hBAFFR:Fas, hBCMA:Fas, or hTACI:Fas were exposed to titrated amounts of the indicated single-chain BAFF-APRIL heteromers which, upon binding, can activate the surrogate intracellular Fas apoptotic pathway that leads to cell death. *A,* effect of FLAG-tagged single-chain ligands cross-linked with a fixed amount of anti-FLAG antibody on the indicated reporter cell lines. *B,* same as *A,* but with Fc-tagged single-chain ligands in the absence of cross-linkers. *C,* same as *A,* but with untagged single-chain heteromers (and FLAG-BBB without anti-FLAG), all in the absence of cross-linker. Data shown in *A–C* are representative of at least two experiments with comparable results.

C-terminal amino acid, which in most TNF family ligands is part of β-sheet H and intimately involved in the structure of the monomer (39), and it is appropriate to wonder whether the addition of a linker at the C terminus of BAFF or APRIL affects structure and activity of these ligands. Single-chain ligands of lymphotoxins α and β were not only competent to bind receptors (Fig. 1) (35) but also recapitulated known receptor-binding specificities for these ligands (Fig. 1) (30, 34). Similarly, single-chain heteromers of BAFF and APRIL retained receptor binding activity, and there were no noticeable differences in constructs when the single copies of BAFF or APRIL were placed in position 1 or 2 (*i.e.* with a linker at the C terminus) or in position 3 (*i.e.* with a natural C terminus). In addition, there was no evidence in the crystal structure of ABB that these short linkers would perturb the structure of BAFF or APRIL in any way. Taken together, these observations suggest that single-chain heteromers provide a valid representation of "classical" homo- or heterotrimers, at least for lymphotoxins and for BAFF and APRIL.

It is worth mentioning that although LTβ is usually reported to be active as heteromer with LTα (34), it was also shown to bind LTβR on its own (46). In line with this later observation, single-chain LT$\beta\beta\beta$ bound LTβR with the same specificity as LT$\alpha_1\beta_2$. The TNF ligand LIGHT binds to LTβR and HVEM, hence it will be interesting to test whether single-chain LT$\beta\beta\beta$ interacts with receptors of the immediate TNF network (TNFR1, TNFR2, HVEM, DcR3, LTβR, and Fas).

A previously characterized APRIL$_2$BAFF$_1$ heteromer activated TACI as efficiently as APRIL in an NF-κB reporter assay, was less potent than either BAFF or APRIL taken alone in B cell proliferation assays, and could be inhibited by BCMA-Fc and TACI-Fc but not BAFFR-Fc (18). Our data are in line with these findings and extend them to APRIL$_1$BAFF$_2$. Briefly, BAA has a similar receptor-binding specificity as AAA, whereas ABB resembles BBB, except for a weaker binding to BAFFR. BAFF has a weaker affinity for BCMA than APRIL (reviewed in Ref. 47), and here we find that ABB is also a weaker binder and activator of BCMA than BAA. The difference is especially
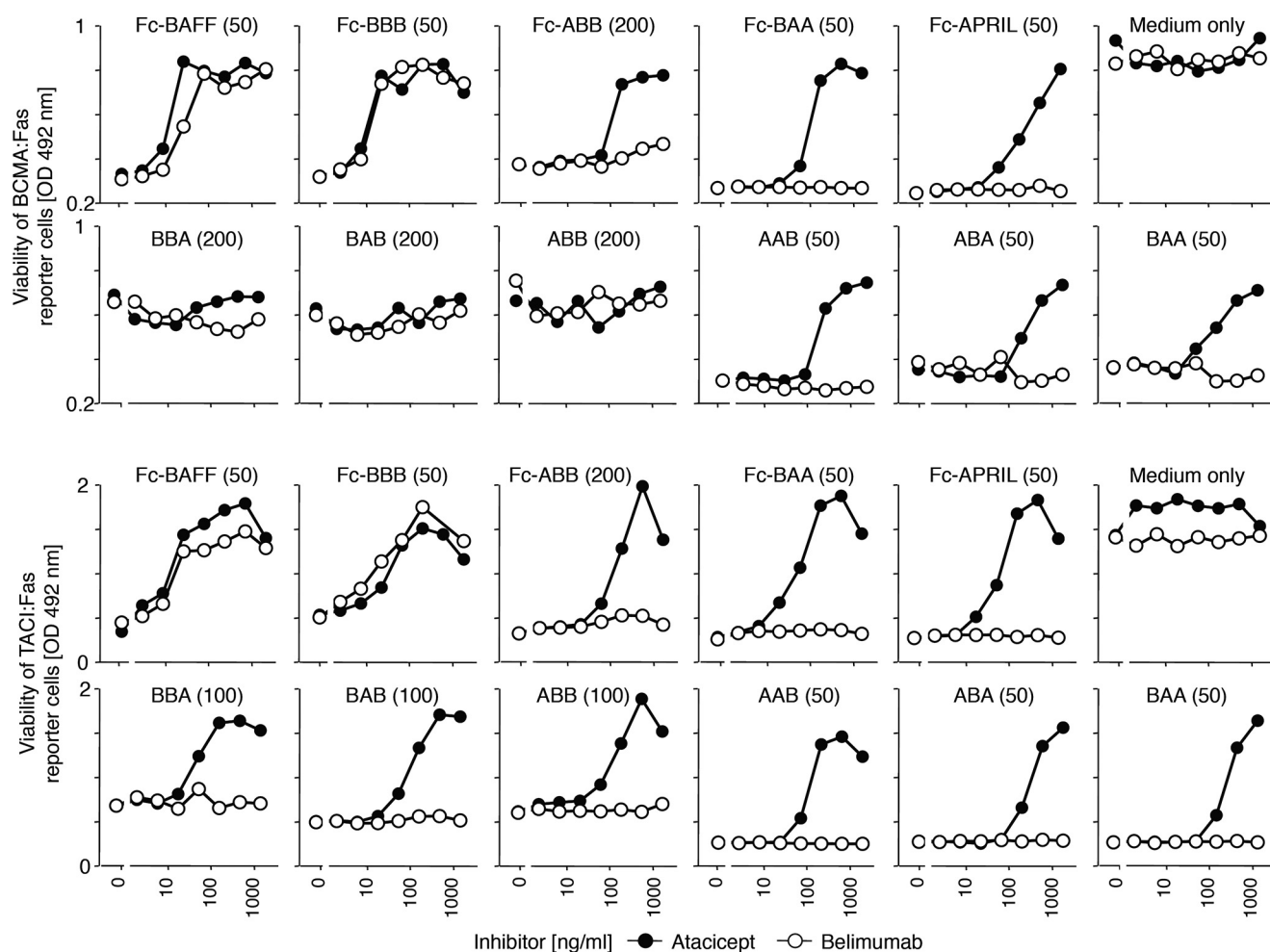
FIGURE 6. **Sensitivity of BAFF-APRIL heteromers of defined stoichiometries to BAFF and APRIL inhibitors.** BCMA:Fas and TACI:Fas reporter cell lines were treated with lethal doses (except for BBA, BAB, and ABB on BCMA:Fas cells; dose in ng/ml indicated in *parentheses*) of the indicated single-chain or conventional BAFF and/or APRIL-containing ligands, in the presence or absence of titrated amounts of hTACI-Ig (atacicept) or anti-BAFF mAb (belimumab). Cell survival was monitored with a cell viability assay. One experiment out of two with similar results is shown.

marked for uncross-linked heteromers, but it is attenuated in the multimerized Fc-fusion constructs. Whether BAFF-APRIL heteromers exist only as soluble trimeric molecules *in vivo* and/or whether they are also multimerized, for example if they exist in membrane-bound forms, remains to be investigated.

In contrast, the binding and activity of BAA and ABB on TACI were more comparable. ABB was severely impaired compared with BBB in all BAFFR binding and BAFFR activation assays, yet they always retained some binding and functional activity on BAFFR, including signaling in primary mouse B cells *in vivo*. The only test system in which ABB showed no activity was in a BAFF-BAFFR competition assay. This same ABB protein retained activity in a similar assay analyzing competition with the BAFF-BCMA interaction (Fig. 4*B*). This indicates that ABB has a lower affinity or avidity than BAFF for BAFFR, a conclusion re-enforced by the examination of the ABB crystal structure that suggests impaired binding to BAFFR. Therefore, a direct consequence of BAFF incorporation into heteromers is to strongly reduce (ABB) or even abrogate (BAA) its ability to activate BAFFR. Heteromers can account for a fair proportion of circulating BAFF in patient sera and may therefore control levels of BAFFR stim-

ulating activity (one study found 0% heteromer in six controls, and 4–87% (average 32%) in 15 patients (19); a second study reported heteromers in 7/89 controls and 24/89 patients with systemic lupus erythematosus, with heteromer concentrations usually lower than those of BAFF, although some subjects had heteromers but undetectable BAFF (48)).

What about stimulation of BCMA and TACI by heteromers? It is predicted that the binding of BAFF, APRIL, or BAFF-APRIL heteromers to TACI will be similar. However, binding to and activation of TACI are distinct events in which ligand oligomerization (*e.g.* BAFF 60-mers or cross-linked APRIL) seems indispensable for TACI activation, at least in primary mouse B lymphocytes (20) and possible in human monocytes (49). Thus, beyond the ability of heteromers to bind to TACI, their ability to stimulate this receptor might well be determined by their capacity or incapacity to oligomerize. Whether endogenous heteromers can oligomerize via the Flap region that is present in BAFF only or via the proteoglycan-binding region that is present in APRIL only is presently an unresolved question. For BCMA, so little is currently known about its activation requirements that it is difficult to speculate whether heteromers will be worse, equal, or better ligands than BAFF or APRIL.
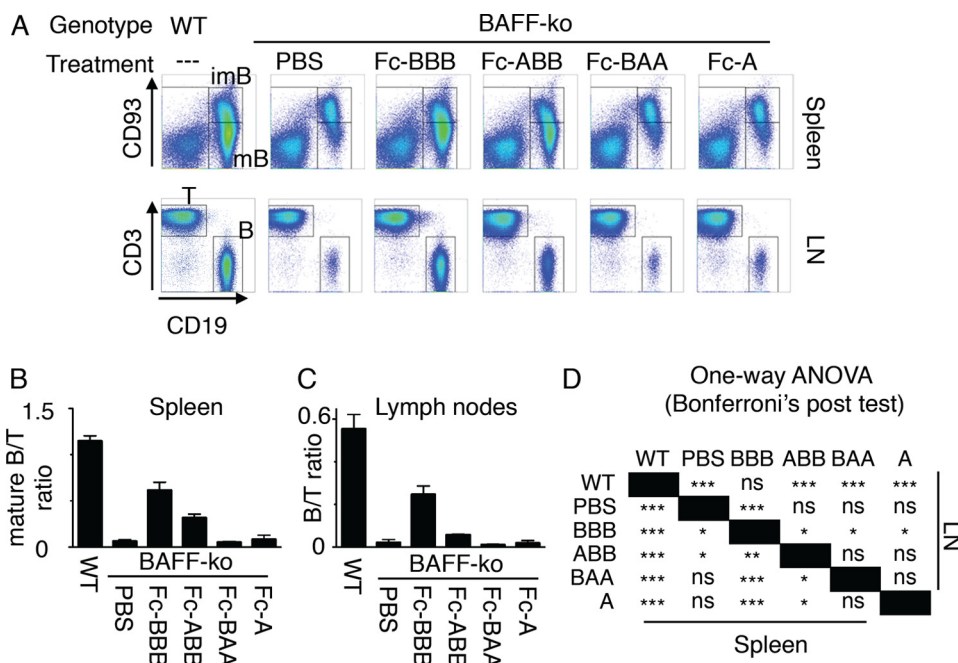
FIGURE 7. Effect of single-chain BAFF-APRIL heteromers in *Baff*-deficient mice. BAFF-KO mice were treated four times over 2 weeks with 1.5 mg/kg of the indicated Fc-tagged conventional or single-chain ligands or with buffer (PBS). Mice were sacrificed 4 days after the last injection. Spleens and lymph nodes (*LN*) were analyzed by FACS for the presence of T cells (*T*) (CD3$^+$), B cells (*B*) (CD19$^+$), immature splenic B cells (*imB*) (CD19$^+$CD93$^+$), and mature splenic B cells (*mB*) (CD19$^+$CD93$^-$). Untreated wild type (*WT*) mice were included as controls. *A,* representative scattergrams of splenic and lymph node cells from mice treated with the indicated Fc ligands. *B,* ratio of mature B cells to T cells in spleens of mice that received the indicated treatments. *C,* ratio of B to T cells in lymph nodes of mice that received the indicated treatments. *D,* results of statistical one-way analyses of variance (*ANOVA*) with Bonferroni's post test multiple comparisons for data shown in *B* and *C. ns,* not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Experiment was performed once with three mice per condition (six mice for WT and six mice for PBS-treated BAFF-KO).

We therefore propose two main hypotheses for the physiological role of BAFF-APRIL heteromers as follows: (*a*) they act as partial functional antagonists of both BAFF and APRIL and down-modulate responses of these ligands on all cells known to respond to BAFF and/or APRIL; (*b*) conversely, they act as dominant negative inhibitors of BAFF signaling on BAFFR, which will affect naive mature B cells. However, they can signal via TACI and BCMA and will therefore support activated and differentiated B cells such as plasmablasts and plasma cells. In the later scenario, heteromers favor antigen-experienced B cells at the expense of naive ones.

Some hints to distinguish between one or the other hypothesis may come from clinical trials comparing single BAFF antagonists (belimumab/Benlysta) to dual BAFF- and APRIL-blocking reagents (*i.e.* atacicept, which is a TACI-Ig fusion protein) (10). Indeed, we show here that ABB and BAA heteromers are efficiently blocked by atacicept but not belimumab. If endogenous heteromers support survival of plasmablasts and plasma cells *in vivo*, then atacicept but not belimumab is expected to affect these cell types; the interpretation will, however, be complicated by the fact that atacicept also inhibits APRIL; the development of specific heteromer antagonists that inhibit neither BAFF nor APRIL will probably be necessary to definitively address the role of heteromers in health and disease. From a mechanistic point of view, the inability of belimumab to interfere with ABB is surprising, as one would expect that it should recognize at least the BB interface. A rational explanation for why belimumab fails to inhibit ABB will probably require crystallization of the belimumab-BAFF complex.

The Flap is a long loop between β-sheets D and E (specifically present in BAFF but no other TNF family ligand) that mediates contact between BAFF trimers and is responsible for the formation of BAFF 60-mers (14, 50). Interestingly, the arrangement of the Flap was not only well resolved in structures of BAFF 60-mers but also in crystals of BAFF 3-mers (Fig. 3*D*) (13, 14, 36–38, 41), suggesting that it may fulfill functions beyond the mere formation of 60-mers.[4] In ABB, the Flap of only one BAFF subunit was similar to that found in BAFF 60-mers, and the second one was differently rearranged, suggesting that a single Flap-Flap interaction might take place between ABB heteromers, although this will be difficult to demonstrate experimentally in solution. Indeed, under conditions where BAFF oligomerizes into 60-mers, 3- and 60-mers are visible in the absence of any defined oligomers of intermediate sizes (14, 50, 51). The Flap region of the C-terminal BAFF subunit of ABB adopted a different, yet well defined conformation that was never observed before (Fig. 3*D*). In the structure, this Flap is also involved in the interaction with an adjacent ABB, but whether and how this affects ABB functionality will require further investigations.

In conclusion, the single-chain ligand technology was successful for studying the properties of BAFF-APRIL heteromers of defined stoichiometry and provided useful tools for the characterization of BAFF and/or APRIL inhibitors. In the future, these reagents will also help to determine whether reagents

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[4] M. Vigolo and P. Schneider, unpublished observations.

used to quantify BAFF or APRIL cross-react with the different forms of BAFF-APRIL heteromers.

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