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Michele Vigolo\textsuperscript{1}, Melissa G. Chambers\textsuperscript{2}, Laure Willen\textsuperscript{1}, Dehlia Chevalley\textsuperscript{1}, Klaus Maskos\textsuperscript{3}, Alfred Lammens\textsuperscript{3}, Aubry Tardivel\textsuperscript{1}, Dolon Das\textsuperscript{1}, Christine Kowalczyk-Quintas\textsuperscript{1}, Sonia Schuepbach-Mallepell\textsuperscript{1}, Cristian R. Smulski\textsuperscript{1}, Mahya Eslami\textsuperscript{1}, Antonius Rolink\textsuperscript{4}, Edith Hummler\textsuperscript{5}, Eileen Samy\textsuperscript{6}, Yves Fomekong Nanfack\textsuperscript{6}, Fabienne Mackay\textsuperscript{7}, Maofu Liao\textsuperscript{2}, Henry Hess\textsuperscript{8}, Xuliang Jiang\textsuperscript{6} & Pascal Schneider\textsuperscript{1}

The B cell survival factor (TNFSF13B/BAFF) is often elevated in autoimmune diseases and is targeted in the clinic for the treatment of systemic lupus erythematosus. BAFF contains a loop region designated the flap, which is dispensable for receptor binding. Here we show that the flap of BAFF has two functions. In addition to facilitating the formation of a highly active BAFF 60-mer as shown previously, it also converts binding of BAFF to TNFRSF13C (BAFFR) into a signaling event via oligomerization of individual BAFF-BAFFR complexes. Binding and activation of BAFFR can therefore be targeted independently to inhibit or activate the function of BAFF. Moreover, structural analyses suggest that the flap of BAFF 60-mer temporarily prevents binding of an anti-BAFF antibody (belimumab) but not of a decoy receptor (atacicept). The observed differences in profiles of BAFF inhibition may confer distinct biological and clinical efficacies to these therapeutically relevant inhibitors.
cells actively participate in the adaptive immune response. Their main function is to produce antibodies that protect against bacterial infections. Antibodies are respectively absent or low in patients with X-linked agammaglobulinemia, who selectively lack B but not T cells, and in patients with common variable immunodeficiency. In both cases, infections of the respiratory and gastro-intestinal tracts are the most common origins and by stromal cells. It binds to three receptors, BAFF selectively lack B but not T cells, and in patients with common ally inhibit a proliferation-inducing ligand (APRIL, also known as TNFRSF13B)-Fc decoy receptor (atacicept), addition-in clinical development, some of which, like a TACI (transmem-styla) was approved in 2011 for the treatment of adult patients BAFF 60-mer. Primary mouse B cells activated in vitro with an least for human BAFF in vitro, into ordered dodecahedrons called TACI is expressed in B cells upon activation and is expressed at higher levels in marginal zone B cells while expression of BCMA forms of BAFF and APRIL, such as BAFF 60-mer, proteoglycan-mer provides the general survival signal for B cells, while other aggregates were inactive in contrast to human BAFF 60-mer, these aggregates were inactive in BAFFR:Fas reporter cells (Fig.2a, b), showing that the tested on BAFFR:Fas reporter cells (Fig.2a, b). Thus, as predicted, both flap mutations prevent 60-mer formation in human BAFF. Lack of active BAFF 60-mer formation in naturally cleaved mouse BAFF may result from an additional exon present in mouse and rat Baff genes that introduces 30 amino acids at the N-terminal of soluble BAFF. This N-terminal extension possibly interfering with 60-mer assembly by steric hindrance (reviewed in ref. 25).

Flap mutations prevent human BAFF to form 60-mers. Naturally cleaved forms of human BAFF eluted from a size-exclusion chromatography column as 3-mers (fractions 15–16), while mouse BAFF formed larger complexes and eluted in fractions 13–14 (Fig. 2a, b). Only WT human BAFF also eluted as BAFF 60-mer in fractions 9–10 (Fig. 2a). All mouse BAFF constructs produced some high-molecular-weight aggregates eluting in the void volume of the column (fractions 7–9) (Fig. 2b), but in contrast to human BAFF 60-mer, these aggregates were inactive when tested for activity on reporter cells (Fig. 2a, b). Thus, as predicted, both flap mutations prevent 60-mer formation in human BAFF. Lack of active BAFF 60-mer formation in naturally cleaved mouse BAFF may result from an additional exon present in mouse and rat Baff genes that introduces 30 amino acids at the N-terminal of soluble BAFF. This N-terminal extension possibly interfering with 60-mer assembly by steric hindrance (reviewed in ref. 25).

Flap–flap interactions are required for BAFF activity. WT human BAFF 3-mer was active on BAFFR:TNF receptor superfamily member 6 (Fas) reporter cells (Fig. 2a). While this activity may be due to re-association into 60-mer, this is unlikely to be the only cause of activity since hBAFF H218A and mBAFF H242A, which cannot form 60-mer, were also active (Fig. 2a, b). Interestingly, hBAFF E223K and mBAFF E247K were inactive when tested on BAFFR:Fas reporter cells (Fig. 2a, b), showing that the two flap mutations tested in this study are not equivalent. This suggests that the flap could serve functions different from that of allowing 60-mer formation.

B cell lymphopenia in E247K flap mutant knock-in mice. A knock-in mouse carrying point mutation E247K in exon 6 of the Baff gene was generated to validate the effect of BAFF flap mutation in a physiologically relevant setting (Supplementary Fig. 2). Endogenous serum levels of circulating BAFF were similar in littermates of Baffwt/wt (WT), BaffE247K (heterozygous), and BaffE247K/E247K (knock-in), but were at background in Baff–/– (knock-out) mice (Fig. 3a). The same was true when receptor binding-competent BAFF was monitored using a recombinant receptor (TACI-Fc) and an antibody (Fig. 3b). We conclude that

Results
Flap mutations do not affect binding of BAFF to receptors. To address the question of how BAFF activates BAFFR, and therefore promotes B cell survival, mutations were designed to inhibit interactions between adjacent flaps. Mutation of histidine 218 (H218A; in mouse: H242A) at the periphery of the flap prevents formation of BAFF 60-mer. Mutation E223K (in mouse: E247K) was also generated because glutamic acid 223 makes a hydrogen bond with lysine 216 at the center of the flap–flap interaction (Fig. 1a).

Recombinant wild type (WT) and flap mutant BAFF, FLAG-tagged or naturally cleaved, were size-fractionated to recover 3-mers, quantified by western blot (Fig. 1b, c) and found to bind immobilized recombinant receptors (BAFFR, TACI, and BCMA) similarly (Fig. 1d). This result was confirmed in a competitive enzyme-linked immunosorbent assay (ELISA) where FLAG-tagged WT or mutant BAFF competed with naturally cleaved untagged WT BAFF for receptor binding. Competition curves were indistinguishable, indicating no major difference in the binding affinities of WT and mutant BAFF to BAFFR (Fig. 1e). WT and mutant BAFF bound identically to endogenous BAFF on BJAB Burkitt lymphoma cells, and to TACI expressed in BAFFR-ko BJAB cells (Supplementary Fig. 1). We conclude that flap mutations selected in this study do not affect binding of BAFF to recombinant receptors, agreeing with crystal structures of BAFF–BAFFR complexes in which flaps make no contact with receptors.

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knock-in mice express and conventionally process BAFF E247K that is then able to bind receptors. Despite the presence of circulating BAFF, the B cell phenotype of knock-in mice resembled that of BAFF knock-out with reduced numbers of CD19+CD93− mature B cells in the spleen, decreased B/T ratio in lymph nodes, but normal numbers of (BAFF-independent) immature CD19+CD93+ splenic B cells (Fig. 3c–f, Supplementary Fig. 3, Supplementary Table 1). There was a non-significant trend (p > 0.05 by one-way Anova) for slightly higher numbers of mature B cells in knock-in mice compared to knock-out, which might reflect a residual activity of either soluble or membrane-bound BAFF E247K (Fig. 3c–f). These results indicate that BAFF E247K cannot, or only very poorly, activate BAFFR in vivo.

In the absence of B cells, circulating levels of BAFF usually rise20,26. We wondered why BAFF levels in B lymphopenic knock-in mice were not higher than in WT and we found that the
Fig. 2 Flap mutations affecting 60-mer formation: one of them additionally affects activity of BAFF 3-mer. Naturally cleaved, untagged human or mouse BAFF, recombinant human BAFF is stable either as 3-mer or 60-mer, requiring for the activity of BAFF (Figs. 2 and 3), and (iv) BAFF activity, like Glu223, are conserved in BAFF of mammals, but not as smaller intermediates such as 6-mer (Fig.2a)19,20,22, (ii) binding to receptors does not require a functional flap (Fig. 1 and Supplementary Fig. 1), (iii) the flap is required for the activity of BAFF (Figs. 2 and 3), and (iv) recombinant human BAFF is stable either as 3-mer or 60-mer, but not as smaller intermediates such as 6-mer (Fig. 2a)19,20,22, we propose that one BAFF 3-mer binds to three BAFFR on a B cell to form an initial complex with no or little signaling ability until two or more complexes are assembled via flap-mediated BAFF–BAFF interactions of BAFF 3-mers contained in these complexes (Fig. 4a). These flap–flap contacts require hGlu223/mGlu247, but not hHis218/mHis242.

Under certain conditions, BAFF–BAFF interactions may be stabilized into even more active 60-mer by additional contacts involving H218, at least for human BAFF (Fig. 4a)20. Residues participating to the core flap–flap interaction that is essential for BAFF activity, like Glu223, are conserved in BAFF of mammals, birds, reptiles, fishes, and sharks, while H218 that is required for 60-mer formation but is not essential for activity is usually not conserved in batrachians and fishes, and may not be relevant in mice and rats (Figs. 2b and 4b). In contrast, the BAFF-like protein of sea lamprey has no flap (Fig. 4b)27.

If the flap really serves the role of connecting BAFF 3-mers in order to activate signaling, as proposed in the model (Fig. 4a), cross-linking antibodies or other strategies to multimerize BAFF should compensate for a deficient flap.

Cross-linking of BAFF 3-mer rescues flap defects in vitro. The activity of size-fractionated FLAG-tagged BAFF 3-mers increased ~100-fold for human BAFF and 10-fold for mouse BAFF (WT or histidine mutants) upon anti-FLAG antibody-mediated cross-linking. The effect was even more pronounced with the glutamic acid mutants (> 1000-fold for human and > 100-fold for mouse). Upon cross-linking, all BAFF gained similarly high activities, although the glutamic acid mutant remained about 3-fold less active than the others (Fig. 4c, d). This could be explained if the cross-linking antibodies cannot arrange BAFF 3-mers in the signaling complex as precisely as via flap–flap interactions. The anti-mouse BAFF antibody 5A8 (and to a lesser extent Sandy-5) was as efficient as anti-FLAG to activate mBAFF E247K assayed on reporter cells (Fig. 4e), or on purified primary mouse B spleenocytes where 5A8 fully corrected the marked signaling defect of mBAFF E247K (Fig. 4f). These results reinforce the conclusion that mBAFF E247K can bind receptors but cannot signal, and additionally demonstrated that a flap defect can be overcome by antibody-mediated cross-linking, in support of the model presented in Fig. 4a.

Anti-BAFF antibodies rescue flap defects in knock-in mice. Knock-in mice express flap-deficient BAFF that is competent for receptor binding but impaired in signaling (Fig. 3). When these mice were treated for 6 weeks with BAFF cross-linking antibodies

A model for two distinct roles of the flap of BAFF. Knowing that (i) two BAFF 3-mers or more can interact via flap–flap interactions, (ii) binding to receptors does not require a functional flap (Fig. 1 and Supplementary Fig. 1), (iii) the flap is required for the activity of BAFF (Figs. 2 and 3), and (iv) recombinant human BAFF is stable either as 3-mer or 60-mer, but not as smaller intermediates such as 6-mer (Fig. 2a)19,20,22, we propose that one BAFF 3-mer binds to three BAFFR on a B cell to form an initial complex with no or little signaling ability until two or more complexes are assembled via flap-mediated BAFF–BAFF interactions of BAFF 3-mers contained in these complexes (Fig. 4a). These flap–flap contacts require hGlu223/mGlu247, but not hHis218/mHis242.

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(5A8 or Sandy-5), mature splenic B cells were significantly 
(p < 0.05 for Sandy-5 and p < 0.01 for 5A8, by one-way Anova) 
increased compared to the control group or to BAFF-deficient 
mice (Fig. 5a, b, d, Supplementary Table 3). B cells also increased in 
lymph nodes where the B to T cell ratio reached half or two 
thirds of WT levels in mice treated with Sandy-5 or 5A8, 
respectively (Fig. 5a, c, d).

These results reveal that knock-in mice contain a latent, inactive 
form of BAFF that can be reactivated in vivo by cross-linking 
antibodies. The repopulation of the B cell compartment proves that 
dendogenous mBAFF E247K protein in knock-in mice can bind and 
activate BAFFR in vivo under adequate conditions, as seen in vitro 
with the recombinant protein. This also reinforces the conclusion 
that the flap of BAFF has a function that is independent of, but as 
crucial as receptor binding for the activity of BAFF. This 
experiment however does not allow addressing the exact mechan-
ism of action of activating antibodies in vivo, which is likely to 
depend both on activation of BAFF activity by cross-linking and on 
the increased half-life of mBAFF bound to antibodies.

**Belimumab and atacicept differentially inhibit BAFF 60-mer.** 
The anti-BAFF antibody, belimumab, is reported to inhibit 
soluble BAFF only. We wondered whether belimumab could
target the flap of BAFF to prevent its signaling function. However, both belimumab and atacicept (TACI-Fc) efficiently inhibited Fc-BAFF, an intrinsically cross-linked (and therefore flap-independent) BAFF, indicating that belimumab is unlikely to target only the flap of BAFF (Fig. 6a, b). However, belimumab did not inhibit naturally cleaved human BAFF, even when belimumab was used at concentrations higher than those of atacicept (Fig. 6c, d).

As naturally processed human BAFF can form 60-mers (Fig. 2a)²², we tested belimumab activity on purified BAFF 60-mer, which belimumab also failed to inhibit (Fig. 6e, f), in line with another report.²⁹ These experiments strongly suggest that belimumab, unlike atacicept, does not (immediately) inhibit BAFF 60-mer.

Belimumab inhibits BAFF 60-mer after dissociation to 3-mer. As BAFF 60-mers are in equilibrium with BAFF 3-mers,¹⁹,²² belimumab should be able to inhibit BAFF 60-mer indirectly by

**Fig. 4** Increase and rescue of BAFF activity by cross-linking. **a** Model of the role of the flap of BAFF for activation of BAFFR signaling: flap–flap interactions for which residue E223 is important mediate interactions between receptor-bound BAFF 3-mers. This promotes BAFFR signaling. Flap–flap interactions for which residues E223 and H218 are both required can further lead to assembly of BAFF 60-mer. **b** Sequence alignment of BAFF from different vertebrates in regions relevant for 3-mer to 3-mer interactions. Residue numbering is for human BAFF. Brackets link residues with symmetric interactions (e.g., K216 of one BAFF 3-mer interacts with E223 of another BAFF 3-mer and vice-versa, see also Fig. 1a), where acidic residues are shown in magenta, basic residues in blue, and the tyrosine that pairs with His218 in turquoise. Hs: Homo sapiens; Mm: Mus musculus; Md: Monodelphis domestica; Gg: Gallus gallus; Ac: Anolis carolinensis; Xt: Xenopus tropicalis; Lc: Latimeria chalumnae; Lo: Lepisosteus oculatus; Om: Oncorhynchus mykiss; Pf: Poecilia formosa; Dr: Danio rerio; Cm: Callorinchus milii; Pm: Petromyzon marinus. **c** Cell viability measured in BAFFR:Fas reporter cells exposed to the indicated concentrations of FLAG-hBAFF WT or mutants in the presence of a fixed concentration of a control antibody (EctoD1) or of an anti-FLAG cross-linking antibody. The experiment was performed twice. **d** Same as panel c, but for FLAG-mBAFF. The experiment was performed twice. **e** Same as panel d, but for FLAG-mBAFF E247K in the presence of a constant concentration of a control antibody (EctoD1), of a cross-linking antibody (anti-FLAG), or of cross-linking anti-mBAFF monoclonal antibodies (5A8 and Sandy-5). The experiment was performed twice. **f** Cell viability of primary splenic mouse B cells cultured for 3 days in the presence of titrated amounts of FLAG-mBAFF WT or E247K, alone or in the presence of fixed amounts of control (EctoD1) or cross-linking (5A8, Sandy-5) antibodies. Each point is the mean ± SEM of technical triplicates. Experiment performed once in this format (and once with less titration points).
Capturing BAFF 3-mers released upon dissociation. To test this hypothesis, BAFF 60-mer was incubated for 3 days at 37°C with or without belimumab or atacicept. BAFF 60-mer alone retained full activity under these conditions (Fig. 6g) and atacicept inhibited BAFF 60-mer both directly and after co-incubation (Fig. 6h). Belimumab did not significantly inhibit BAFF 60-mer when used directly, but could do so, after a co-incubation of 3 days, although still less efficiently than atacicept (Fig. 6i). In another experiment, belimumab was incubated with an excess of BAFF 60-mer, with the expectation that binding to BAFF 60-mer or to BAFF 3-mer would differently shift the size of belimumab. In this experiment, the elution position of belimumab was indeed shifted, but remained smaller than that of BAFF 60-mer still present in the mixture (Supplementary Fig. 5). Taken together these results indicate that belimumab cannot inhibit BAFF 60-mer directly, but can eventually do so when BAFF 60-mer dissociates into 3-mers.

3D elucidation of the mechanism of action of belimumab. The antigen-binding fragment (Fab) of belimumab was produced by digestion with papain, while BAFF 3-mer was expressed in prokaryotic cells. To prevent association of BAFF 3-mer into 60-mer, mutation H218A was introduced into the flap region, which did not grossly affect the affinity of belimumab for BAFF (Supplementary Fig. 6). BAFF 3-mer or the Fab taken alone had roughly equal native sizes (Fig. 7a–c), but formed a larger, well-defined complex when BAFF was mixed with an excess of belimumab Fab (Fig. 7d). Some free Fab was detected when the complex was reanalyzed by size-exclusion chromatography (Fig. 7e), suggesting that the Fab was not very tightly bound to BAFF. As expected, the high-molecular-weight complex contained both BAFF and the Fab when analyzed by reducing or non-reducing SDS-PAGE (Fig. 7f). The BAFF–Fab complex was analyzed by both electron microscopy and crystallography (Supplementary Fig. 7, Supplementary Table 4). Negative stain electron microscopy images often revealed structures with three arms on which the crystal structure could readily be superimposed (Fig. 7g, h). The complex sometimes also appeared as L-shaped, which could be interpreted either as a complex with 3 Fab, one of which would stand perpendicular to the image plane, or as a BAFF 3-mer bound to only two Fab (Fig. 7i, j). Incomplete complexes with a single Fab bound to a BAFF 3-mer could also be seen (Fig. 7k). This less abundant form was likely excluded from crystals where only BAFF bound to three Fabs could be observed. Interestingly, the relative instability of the BAFF–Fab complex
Belimumab differs from atacicept in that it does not inhibit BAFF 60-mer promptly. BAFF-responsive BAFFR:Fas reporter cells were treated overnight with titrated amounts of the indicated forms of BAFF, in the presence or absence of the inhibitors atacicept or belimumab at fixed concentrations. Cell viability was then monitored. A, B Inhibition of Fc-BAFF by atacicept (A) or belimumab (B). The experiment was performed 3 times. C, D Naturally processed hBAFF in supernatants of transfected 293 T cells exposed to atacicept at 0 or 5 ng/ml (C) or belimumab at 80 ng/ml (D). The experiment was performed twice. E, F Inhibition of Fc-BAFF by atacicept (E) or belimumab (F). The experiment was performed 3 times. G BAFF 60-mer was titrated in medium, then incubated in medium for 3 days at 37 °C, then added to reporter cells. Alternatively, BAFF 60-mer was titrated in medium just before the assay (fresh). H, I BAFF 60-mer was titrated in medium without or with atacicept (H) or belimumab (I) for 3 days at 37 °C. Samples without inhibitors received inhibitor or not after 3 days of incubation. Samples were then added to reporter cells. The experiment was performed twice. Panels A, B, E, and F show the mean ± SEM of 5 replicates, panels C and D of duplicate, and panels G, H, and I of triplicate measures. The condition without inhibitor is the same in panels A and B, in panels C and D, in panels E and F, and in panels G, H, and I. See also Supplementary Fig. 5.

was suggested by the observation that the purified complex was almost as active as BAFF alone on reporter cells, which could be explained either by the existence of a small proportion of free BAFF, or by competition of high-affinity BAFFR with Fab for a common binding site on BAFF (Fig. 71).

A closer look at the structure of the BAFF–belimumab Fab complex showed that both heavy and light chains of belimumab contacted BAFF over its entire height, forming two distinct interfaces with the receptor-binding site (664 Å²) and the flap region (333 Å²), for a total average surface area of 995 Å² (963–1014 Å² for the six copies of the asymmetric unit) (Fig. 8a). In contrast, in a model of TACI bound to BAFF, the receptor bound to a much smaller region of BAFF (Fig. 8b) that was clearly separated from the flap–flap interaction region mediating BAFF 60-mer formation (Fig. 8c).

The BAFF–Fab complex is the first structure in which BAFF 3-mers make no contact with adjacent BAFF 3-mers via flap–flap interactions. Nevertheless, the conformation of the flap loop was virtually identical to that of a flap engaged in 3-mer to 3-mer contacts, indicating that its conformation is constitutive and not induced by binding (Fig. 8d). In addition, mutation H218A did not grossly alter conformation of the flap (Fig. 8d). Belimumab made important contacts with a small cavity of BAFF that normally accommodates the DXL loop motif present in all natural receptors of BAFF and that is essential for receptor–ligand interactions30. In fact, one of the variable loops of the heavy chain of belimumab contained a DXL (DLL) motif that perfectly mimicked the receptor-binding site of BAFF. Taken together, these data show that belimumab hijacks the natural receptor-binding site of BAFF via a DXL motif in its heavy chain, but that the bulky nature of the Fab and the additional contacts made by the light chain with BAFF prevent interactions with BAFF 60-mer.

**Atacicept binds to the surface of intact BAFF 60-mers.** BAFF 60-mer migrated with a high molecular weight by size-exclusion...
When BAFF 60-mer was mixed with an excess of atacicept on ice, a heavy precipitate formed, and the supernatant only contained excess soluble atacicept with no trace of complex (Fig. 9c). However, when resuspended in buffer, the precipitated complex reversibly dissolved at room temperature and re-precipitated on ice (Fig. 9d). The atacicept–BAFF 60-mer complex could therefore be isolated by size-exclusion chromatography at room temperature (Fig. 9e, f).

Analysis of BAFF 60-mer by electron microscopy revealed a well-organized, capsid-like structure of about 220 Å diameter (Fig. 9g, pictures on the left)\(^{19}\). In the presence of atacicept, these particles enlarged to a diameter of about 340 Å, indicating that they had been coated with atacicept without affecting assembly of the underlying BAFF 60-mer (Fig. 9g, pictures on the right). We conclude from these studies that although atacicept and belimumab share common features for BAFF binding (the DXL motif), they differ by their binding ability to un-dissociated BAFF 60-mer.
Fig. 8 Prominent features of the BAFF 3-mer-belimumab Fab complex. The structure of BAFF H218A (3-mer) in complex with the Fab of belimumab was solved. The experiment was performed once. A model of the cysteine-rich domain 2 of TACI bound to BAFF was obtained by superposing the structure of one BAFF monomer to the APRIL-TACI complex (PDB-ID 1XU). a Structure of one BAFF monomer (pale yellow) to one belimumab Fab (heavy chain in black, light chain in green). The flap of BAFF is shown in darker yellow. b Model of TACI bound to BAFF. c Model showing the position of the adjacent BAFF monomer if a flap-flap interaction would have occurred. The model was built by superimposing two relevant BAFF monomers from the BAFF 60-mer structure (PDB-ID 1OQE) to one BAFF monomer of the BAFF-belimumab Fab complex. d Superimposition of the flap of a BAFF from the BAFF 60-mer structure (pale purple) (PDB-ID 1OQE) and a flap from the BAFF H218A-belimumab Fab complex (orange). Residues K216 and E223 that make important contacts with E223 and K216 of an adjacent BAFF from the BAFF 60-mer structure are shown. e Detail of the receptor-binding pocket of BAFF, showing interaction with the DXL motif of belimumab (D101, L103). The DXL motif of TACI (D80, L82) (PDB-ID 1XU) is superimposed. Y206 and R265 of BAFF are also shown. f The structure of the BAFF-Fab complex of panel a was merged to that of the flap-mediated BAFF-BAFF interaction of panel c. The large clash area between both structures suggests that belimumab cannot access its binding site in BAFF 60-mer.

Potential impact of distinct anti-BAFF drugs specificities. In the present study, the structural basis for the distinct inhibitory mechanisms of BAFF 60-mer by belimumab and atacicept was characterized. This is not the only difference between belimumab and atacicept regarding their substrate specificity: atacicept, but not belimumab, also inhibits APRIL, and heteromers of BAFF and APRIL. Whether these differences positively or negatively impact the therapeutic action of these drugs is unknown. This will depend on whether drug targets are expressed or not in the pathological context, and also on how detrimental pathogenic cells (e.g., autoreactive or pro-inflammatory cells) and useful protective cells (e.g., normal plasma cells or regulatory cells) overlap with their requirements for potentially distinct sets of survival factors and for the set of receptors (BAFFR, TACI, and BCMA) used to respond to them. For example, if a subset of autoreactive plasma cells are pathogenic in human SLE, and these cells require either BAFF or APRIL, like normal mouse plasma cells, atacicept could be superior to belimumab. But if this same pathogenic cell subset would turn out to require BAFF only, atacicept could induce an unnecessary immunodeciency. With regard to BAFF 60-mer, about which almost nothing is known in humans, much work is still needed to understand whether and where this complex occurs, and for which physiological or pathological functions. Also, the different mechanisms of BAFF 60-mer inhibition characterized in this study could be irrelevant in vivo if BAFF 60-mer dissociates into 3-mers before it can act on target cells, or crucial if newly synthetized BAFF 60-mer can reach its target before dissociation.

Modulation of TNF family ligand activity by oligomerization. It has long been recognized that membrane-bound and soluble TNF differentially activate TNFRSF1A (TNFR1) and TNFRSF1B (TNFR2) and fulfill different functions in inflammation and immunity to mycobacteria. Similarly, membrane-bound and soluble TNF ligand superfAMILY member 6 (FasL) have different capacities to induce either apoptosis or non-deadly functions such as cell migration (reviewed in ref.36). Membrane-bound ligands can only deliver signals in a cell-to-cell contact but can activate receptor functions more efficiently than soluble ligands. In contrast, soluble ligands can act systemically but require receptors responsive to such soluble ligands. The plasma membrane is believed to cluster membrane-bound ligands, a function that can be mimicked when soluble ligands are either cross-linked or engineered to contain two trimers within a single molecule. Ectodysplasin-A (EDA), the only TNF family ligand containing a collagen domain, must be processed to a soluble form to fulfill its functions in the embryonic development of skin-derived appendages. In this case, it is the collagen domain that mediates an intrinsic oligomerization to make soluble EDA efficient activators of their oligomerization-sensitive receptor. BAFF is another ligand with an intrinsic propensity to oligomerize, but this time as a 60-mer. BAFF 60-mer, or membrane-bound BAFF, might be...
required to activate TACI and possibly BCMA. In contrast, BAFFR can also be stimulated by BAFF 3-mer and, in that respect, resembles TNFR1 activated by soluble TNF. We now suggest that the flap of BAFF clusters individual complexes of BAFF 3-mer/BAFFR to form multimers endowed with the capacity to initiate relevant B cell survival signals in vitro and in vivo. Clustering of individual BAFF-BAFFR signaling units probably represents the primary and sine qua non function of the flap of BAFF, while the capacity of the flap to assemble BAFF 60-mer could be a secondary improvement of this oligomerizing function.

In summary, binding and activation of BAFFR by BAFF are distinct steps. With regard to BAFF-directed therapies, the flap of BAFF should be considered as a target potentially as good as the receptor-binding site for BAFF inhibition. Conversely, stimulating BAFF activity by promoting oligomerization could prove beneficial in certain immune deficiencies. Finally, the flap of BAFF not only controls BAFF signaling (via 3-mer to 3-mer interactions) and BAFF potency (via 60-mer formation), but can also interfere with the binding and action of anti-BAFF drugs.

Methods

Animals. Baff−/− mice were as described. C57BL/60aHsd mice were purchased from Envigo (Horst, Netherlands, stock # 057) and housed in a specific pathogen-free animal facility. BAFF E247K knock-in mice were generated according to standard procedures. Briefly, embryonic stem cells (129 SvEv) were electroporated with a construct containing the E247K mutation (GAG CTG → AAG CITG) in exon 6 of Baff and a floxed NeoR cassette inserted in introns 6–7 and screened by PCR and Southern blot for homologous recombination events (Supplementary Fig. 2). Recombined embryonic stem cells were injected into C57BL/6 blastocysts followed by transfer into the uterus of pseudo-pregnant recipients. Progeny of chimeric mice that transmitted the mutation was backcrossed for 4 generations onto the C57BL/6 background, then bred to B6.C-Tg(CMV-cre)1Cgn/J mice (sn#00654, The Jackson Laboratory) to remove the NeoR cassette. Heterozygous mice were bred to obtain cohorts of BaffE247K/E247K, Baffwt/E247K and BaffE247K/+ littermates, or bred to Baff−/− to obtain cohorts of BaffE247K−/−, Baffwt/−, BaffE247K−/− and BaffE247K−/− littermates. Experiments were performed according to guidelines and under the authorization of the Swiss Federal Food Safety and Veterinary Office (authorization 13706.0 to PS).

Genotyping. Ear biopsies were digested for 2–16 h at 55 °C in 50 μl of the DirectPCR Lysis Reagent (Peqlab, 31-102-T) plus 2 μl of Proteinase K (Roche), followed by 45 min at 85 °C. Supernatants of BAFF-E247K ki lysates were used for PCR using primers fwd 5′-ACCCTGTCCCCAGTATTCGA-3′ and rev 5′-TAA-GAGGTCGAGGTCGCC-3′, with 30 cycles of 94 °C (7 s)/58 °C (20 s)/72 °C (40 s). Supernatants of BAFF-ko lysates were used for PCR using primers wt shared 5′-CAAGTTGATGTCCTGACCAAGGACAC-3′ and neo 5′-TGGACGGGTCTTGGACAGCCTGACTG-3′, with 30 cycles of 94 °C (7 s)/60 °C (20 s)/72 °C (20 s). The screening of recombedinated ES cells, genomic DNA was amplified with primers neo fwd 5′-CTCTGTACCATGGCTTGGAC-3′ and BAFF rev 5′-GTGGAACAGATAGAACCCTGTTCCGATGTATTCA-3′, with 3 cycles of 95 °C (30 s)/64 °C [ramp-0.5 °C/cycle] (30 s)/68 °C [ramp-0.5 °C/cycle] (30 s).
(2 min), followed by 30 cycles of 95 °C (30 s)/54 °C (30 s)/68 °C (2 min), using TaKaRa LA polymerase (TaKaRa) at 3 U/ml.

Reagents and cell lines. The following antibodies were used: mouse IgG1 anti-FLAG M2 and biotinylated M2 (Sigma Aldrich F3165 and F2991), goat IgG 852 anti-mouse BAFF (R&D AF2106), biotinylated mouse IgG anti-human BAFF huBR9.1 (Adipogen, Liestal, Switzerland, AG-20B-0016B), rat IgG1 anti-mouse BAFF SAA45, mouse IgG1 anti-mouse BAFF Sandy-2 and Sandy-545, rat IgM anti-human BAFF Buffy-25, and mouse IgG1 anti-ELDA EctoD142. Human IgG1 anti-human BAFF (H218A) were obtained from the Pharmacy of Lausanne University Hospital (CHUV). Hb-BAFF-Fc was from Adipogen (AG-40B-0027) and hTACI (aa 31–110)–hlg Ig Fc (aa 245–470, L28E, A335S, P354S) (atacetap) was described by Merck, KGaA. HEK 293, HEK 293 T, Jurkat, and BJAB cells were obtained from late Jürg Tschopp (University of Lausanne). CHO-S cells were from Thermoscientific (A1155701). Reporter cells Jurkat-JOM2-hBAFF-Fas Fls 21,11,14,14 were described as and grown in RPMI supplemented with 10% fetal calf serum (FCS). HEK 293 T cells were grown in DMEM 10% FCS. BJAB and BJAB–TACI cells45,46 were grown in RPMI supplemented with 10% FCS. Cell lines were tested for mycoplasma using MycoAlert mycoplasma detection kit (Lonza, LTOT-318) and found to be negative. As the identity of CHO-S, HEK 293, HEK 293 T, Jurkat and BJAB cells does not impact on result interpretation, these cell lines were not authenticated. The identity of BJAB cells was confirmed by microsatellite sequencing (cell line typing service, Microsynth, Balgach, Switzerland).

Immunoglobulin-depleted fetal calf serum. A volume of 500 ml of FCS was depleted from immunoglobulins by repeated passages (usually eight times) at 4 ml/min on a 5 ml Protein A-Sepharose column (GE Healthcare), until the fraction of immunoglobulins bound to the column became negligible. Immunoglobulin-depleted serum was sterilized by filtration at 0.2 μm.

Purification of Fc-containing recombinant protein. Fc-containing proteins (from plasmids listed in Supplementary Table 5) were produced from stable clones of HEK 293 or CHO-S cells. Cells were grown in serum-free OptiMEM medium or in DMEM/F12 (1:1, v/v) medium supplemented with 2% of immunoglobulin-depleted FCS. Proteins in conditioned supernatants were affinity-purified on 5 ml (or 1 ml) Protein A- or Protein G-Sepharose column (GE Healthcare), eluted with 50 mM citrate-NaOH pH 2.7 and neutralized with appropriate amounts of 1 M Tris–HCl pH 8.4. Proteins were concentrated, and buffer exchanged to 30 kDa cutoff centrifugal devices, then sterilized by filtration at 0.2 μm, quantified by absorbance at 280 nm using theoretical molar extinction coefficients and stored at −70 °C until use45,46.

Purification of His-tagged BAFF. Esherichia coli M15 pREP4 bacteria expressing His-tagged hBAFF H218A (3-mer) and His-tagged hBAFF (60-mer)45 in early logarithmic phase of growth were grown overnight at 18 °C in L-Broth medium supplemented with 1.5% of isopropyl-thiogalactoside. Bacteria were harvested, lysed by sonication in 30 ml of 10 mM Tris–HCl pH 8, 0.5 M NaCl per liter of culture and insoluble material was removed by centrifugation (15 min, 17,000 × g). Supernatants were loaded (up to three liter equivalent of culture) on a 5 ml cation-exchange-Sepharose column (GE Healthcare) pre-loaded with 0.5 M NaCl and equilibrated in lysis buffer. The column was washed with two volumes of 10 mM Tris–HCl pH 7.4, 150 mM NaCl (TBS), five volumes of TBS 50 mM imidazole, two volumes of TBS, and eluted with three volumes of TBS, 50 mM ethylenediaminetetraacetic acid (EDTA). Proteins were concentrated (30 kDa cutoff) and fractionated by size-exclusion chromatography on a Superdex 200 column as described under ‘‘size-exclusion chromatography.’’ Fractions corresponding to BAFF 3-mer or BAFF 60-mer were filter-sterilized, quantified by absorbance at 280 nm and stored at −70 °C.

Generation of the Fab fragment of belimumab. Quantity of 120 mg of belimumab was mixed with 500 μl of immobilized papain beads (ThermoScientific) in 3 ml of 20 mM NaCl, 20 mM Na-phosphate pH 7.5, 10 mM EDTA, and digested for 4 days at 37 °C. At the end of the incubation, beads were removed and the Fab was recovered in the flowthrough of a Protein A affinity column. Fab were concentrated on 10 kDa cutoff centrifugal devices, then size-fractionated on a Superdex 200 Increase column in 20 mM HEPES pH 7.5, 130 mM NaCl.

Relative affinity of belimumab Fab for Fc-BAFF WT and H218A. Quantity of 300 μg of belimumab Fab in 300 μl of 0.1 M Na-borate pH 8.8 was biotinylated for 2 h at 4 °C with 3 μl of sulfo-N-hydroxysuccinimide-LC-biotin (Pierce, 21335) at 30 μg/ml in DMSO. Reaction was terminated by addition of 10 μl of 1 M NH4Cl, and buffer was exchanged to PBS using a 30 kDa cutoff centrifugal device. Fc-hBAFF (WT or H218A, 100 μl) was coated at 1 μg/ml in PBS in an ELISA plate. After blocking, 50 μl of soluble Fab-BAFF (WT or H218A) were added at 2-fold the desired final concentration, followed by the addition of 50 μl of 100 ng/ml biotinylated Fab of belimumab and immediate mixing. After washing, bound Fab was detected with horseradish peroxidase-coupled streptavidin.
Receptor-ligand interaction ELISA. For the direct-binding ELISA, BAAF-Fc, BCMA-Fc, or TACI-Fc (human or mouse) were adsorbed at 1 μg/ml in PBS overnight at room temperature into 96-well immunoplates. The following steps were then performed, with incubations at 37 °C: blocking (in PBS, 4% powdered skimmed milk, 0.5% Tween-20), washing (in PBS, 0.05% Tween-20), incubation with FLAG-tagged BAFF 3-mers of the corresponding species at the indicated concentrations for 1 h (in incubation buffer: PBS, 0.4% milk, 0.05% Tween-20), washing, incubation with biotinylated anti-FLAG at 0.5 μg/ml in incubation buffer for 1 h, washing, incubation with horseradish-coupled streptavidin at 1/4000 dilution in incubation buffer, washing, incubation with 100 μg/ml of 3,3',5,5'-tetramethylbenzidine in 50 mM citrate-phosphate pH 5, 10% DMSO, 0.05% perborate until color development, addition of 1/3 volume of 0.1 M HCl, and enzyme reading at 405 nm. For the competitive ELISA, titrated amounts of naturally cleaved (untagged) wild-type BAFF were added to adsorbed BAAF-Fc for 30 min, followed without intermediate washing steps by a fixed and non-saturating concentration of FLAG-tagged BAFF 3-mers (10 ng/ml for human and 50 ng/ml for mouse). Detection with biotinylated anti-FLAG was performed as described for the direct-binding ELISA.

Electron microscopy data acquisition and image processing. Negatively stained specimens were prepared following an established protocol with minor modifications. Specifically, 2.5 μl of sample was applied to glow-discharged copper electron microscopy (EM) grids covered with a thin layer of continuous carbon film, and the grids were stained with 2% (w/v) uranyl formate. The sample concentrations of BAFF 60-mer, BAFF 60-mer bound with atacicept, and BAFF 3-mer bound with Fab were 23, 20, and 8.8 μg/ml, respectively. These grids were imaged on a Tecnai T12 electron microscope (FEI) operated at 120 kV, negative staining using 67,000x magnification with a 4 × 4 k CCD camera (UltraScan 4000, Gatan), corresponding to a calibrated pixel size of 1.68 Å on the specimen level. The EM data were processed using SAMUEL and SamViewer. Negative stain EM images were binned over 2 × 2 pixels for further processing, yielding a pixel size of 3.36 Å. Particle picking was performed using a semi-automated procedure. 2D classification of selected particle images was carried out with ‘samclass.py’, which uses SPIDER operations to run 10 cycles of correspondence analysis, K-means classification, and multi-reference alignment.

Statistics. Group sizes for animal experiments were chosen to detect 40% differences between conditions, with variation coefficients of 15–20% (4 animals/group). Animals were assigned randomly to groups, except for males and females that were distributed equally between groups. Analyses were not blinded. Normal distribution of data was confirmed by normality tests using Prism (D’Agostino and Pearson test for n ≥ 8, Kolmogorov–Smirnov test for n = 6 or 7), or was assumed for smaller size groups. One-way analysis of variance with Bonferroni’s multiple comparison tests was used to compare selected groups using Prism. One-way analysis of variance assumes that the standard deviation of the different groups is equal, an assumption that was not always met according to Bartlett’s test for equality of variance. But as this assumption is of little importance when group sizes are (almost) equal, this was ignored.

Data availability. The authors declare that the data supporting the findings of this study are available within the article, in its supplementary information files, as a dataset, or are available upon reasonable requests to the authors. The structural datasets are available in the Protein Data Bank, www.pdb.org, under the PDB-ID code 6ERX.
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Acknowledgements
We are grateful to Susan Kalled and Linda Burky (Biogen, Boston) for providing BAFF+/- mice, and to Anne-Marie Méritall (University of Lausanne) for assistance with creation of knock-in mice. This work was supported by grants from the Swiss National Science Foundation (to P.S.), and by a research grant from EMD Serono, a subsidiary of Merck, KGaA (to P.S.).

Author contributions
P.S. conceived the experiments and generated protein complexes for EM and crystallography, D.D., P.S., and E.H. generated knock-in mice, M.G.C. and M.L. performed and analyzed EM. K.M. and A.L. contributed experimental ideas and key reagents to perform them. P.S. wrote the paper with the help of M.V. All the authors reviewed the results and approved the final version of the manuscript.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-018-03323-8.
Competing interests: A.L. and M.K. are employees of Proteros biostructures GmbH. E. S., Y.F.N., and X.J. are employees of EMD Serono. H.H. is employee of Merck, KGaA. The remaining authors declare no competing interests.

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