Unique and redundant roles of mouse BCMA, TACI, BAFF, APRIL, and IL-6 in supporting antibody-producing cells in different tissues

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Abstract

Antibody-producing plasma cells fuel humoral immune responses. They also contribute to autoimmune diseases such as systemic lupus erythematosus or IgA nephropathy. Interleukin-6 and the tumor necrosis factor (TNF) family ligands BAFF (B cell-activating factor) and APRIL (a proliferation-inducing ligand) participate in plasma cell survival. BAFF binds to three receptors, BAFFR (BAFF receptor), TACI (transmembrane activator and CAML interactor), and BCMA (B cell maturation antigen), while APRIL binds to TACI, BCMA, and proteoglycans. However, which ligand–receptor pair(s) are required to maintain plasma cells in different body locations remains unknown. Here, by combining mouse genetic and pharmacological approaches, we found that plasma cells required BCMA and/or TACI but not BAFFR. BCMA responded exclusively to APRIL, while TACI responded to both BAFF and APRIL, identifying three self-sufficient ligand–receptor pairs for plasma cell maintenance: BAFF–TACI, APRIL–TACI, and APRIL–BCMA. Together, these actors accounted for 90% of circulating antibodies. In BAFF-ko mice, the reduction of plasma cells upon APRIL inhibition indicated that APRIL could function in the absence of BAFF–APRIL heteromers. No evidence was found that in the absence of BCMA and TACI, binding of APRIL to proteoglycans would help maintain plasma cells. IL-6, alone or together with BAFF and APRIL, supported mainly splenic plasmablasts and plasma cells and contributed to circulating IgG but not IgA levels. In conclusion, survival factors for plasma cells can vary with body location and with the antibody isotype that plasma cells produce. To efficiently target plasma cells, in particular IgA-producing ones, dual inhibition of BAFF and APRIL is required.

Significance Statement

Antibodies are important for the control of microorganisms and foreign molecules in the context of adaptive immunity but can also be detrimental when directed against self-antigens. B cell-activating factor (BAFF), a proliferation-inducing ligand (APRIL), and their receptors are critical for the generation and function of B cells and their fully differentiated stage of antibody-producing plasma cells. They are targeted in the clinic to modulate antibody and autoantibody production. However, BAFF and APRIL form a complex system of three ligands (when considering their heteromers) and three receptors plus proteoglycans. Therefore, it is clinically relevant to understand the unique and redundant contributions of each pair of these molecules, as described in this study, to better interpret results of pharmacological interventions.

Introduction

Antibodies of humoral immune responses are produced by terminally differentiated B lymphocytes called plasma cells. The half-life of antibodies in serum is relatively short, in the range of 3 to 21 d. In order to maintain humoral immunity against commensals and pathogens long after their encounter, some of the induced plasma cells are maintained over months to years as long-lived cells, while others are short-lived and observed only during few days. Both types of plasma cells are generated within secondary lymphoid organs such as the spleen, lymph nodes (LN), and Peyer's patches which serve as inductive sites for adaptive immunity. While many short-lived plasma cells are found within these inductive sites as well as the intestinal lamina propria, long-lived plasma cells typically home to the bone marrow (BM) or the gut (1-6). An estimated 70% of all plasma cells are found within the intestinal lamina propria, responsible for continuous protection of mucosal surfaces, mostly via secretion of IgA into the gut lumen. IgA^+ plasma cells similarly protect the mucosal surfaces of the lung. The remaining plasma cells are enriched in the BM, secondary lymphoid organs, and peritoneal cavity and are responsible for maintaining serum IgM, IgG, and IgA titers (7-9). While antibodies have an important protective function against infectious agents, antibodies reactive against self-antigens have also been recognized as key drivers of autoimmune disease. Thus, a better understanding of plasma cell lifespan regulation has important therapeutic relevance.

While plasma cell-intrinsic factors are involved in regulating plasma cell longevity, much research has focused on cell-extrinsic factors such as B cell-activating factor (BAFF), a proliferation-inducing ligand (APRIL), IL-6, TNF, and CXCL12. These cytokines are constitutively produced within so-called survival niches, like in the BM where plasma cells can home to in order to increase their longevity (*10-16*). Currently, the relative importance of these various survival factors and their receptors in plasma cell survival is unclear in vivo, in part due to the role of factors like BAFF in B cell development and naive B cell maintenance. Also poorly characterized is whether these factors differentially regulate plasma cell pools in different organs.

BAFF and APRIL, two ligands of the TNF family, are type II transmembrane proteins that can be released as soluble trimers upon processing at a furin consensus cleavage site (*17*). They can be expressed individually or form heteromers into which one APRIL subunit can bind to two BAFF subunits, or vice versa (*18, 19*). BAFF, APRIL, and their heteromers share two receptors, transmembrane activator and CAML interactor (TACI) and B cell maturation antigen (BCMA). TACI binds both ligands with similar affinities while BCMA has a higher affinity for APRIL. BAFF can additionally bind to BAFF receptor (BAFFR) (*17*). Interaction of these cytokines and their receptors leads to activation of nuclear factor kappa B (NF-kB) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathways, production of anti-apoptotic Bcl-2 family members, and eventually survival of B cells and plasma cells (*20*).

BAFFR is expressed on immature B cells in the BM and on transitional and mature B cells in secondary lymphoid organs leading to activation of prosurvival signals which rescue these cells from premature death (*21-24*). Maintenance of mature peripheral B cells depends exclusively on BAFF acting through BAFFR (*21, 25*). Expression of functional B cell receptors up-regulates the expression of BAFFR on immature and transitional B cells (*26*). TACI is highly expressed on transitional and marginal zone B cells, consistent with its important role in T cell-independent B cell responses (*21*). Nevertheless, TACI-ko mice have an elevated number of B cells and splenomegaly which indicates that TACI is also a negative regulator of B cells (*27*). BCMA is expressed in germinal center B cells, in $CD38^+$ plasmablasts, and in terminally differentiated plasma cells (*21, 28, 29*). The survival of long-lived plasma cells in the BM is impaired in BCMA-ko mice (*29*), APRIL-ko mice have reduced IgA levels (*30*), and an elegant study showed that the maintenance of IgG-

positive plasma cells in BM and spleen required both BAFF and APRIL (*12*). BAFF and APRIL are implicated in several pathological conditions such as autoimmune disorders (*31-33*), asthma (*34-38*), and cancer (*39-44*). Consequently, BAFF alone, APRIL alone, or both BAFF and APRIL are targeted in clinical trials for treating autoimmune patients with systemic lupus erythematosus or IgA nephropathy (*45, 46*). However, which ligand precisely engages which receptor to support plasma cell survival *in vivo* has not been systematically studied and remains unknown. Here, we used genetic and pharmacological approaches to answer this question and found that plasma cells in the BM and in the intestine rely mainly on three ligand/receptor pairs: APRIL-BCMA, BAFF-TACI, and APRIL-TACI. Only by blocking all three interactions did plasma cells almost fully disappear and did IgA, IgM, and IgG levels strongly decrease. In addition, IL-6 contributed to maintain IgG and, to a lesser extent, IgM levels by acting on splenic plasmablasts and plasma cells, but unlike BAFF and APRIL did not regulate IgA levels.

Results

Pharmacological tools to block mouse BAFF, mouse APRIL, and their heteromers.
To inactivate BAFF and APRIL, we used Centotto-1, a monoclonal mouse IgG1 that targets the receptor-binding site of mouse APRIL (*47*), and Sandy-2, a mouse IgG1 that blocks mouse BAFF (*48*) (Figure 1A). The fine specificities of Centotto-1 and Sandy-2 were evaluated using BCMA:Fas reporter cells that undergo Fas-mediated killing upon stimulation with either BAFF, APRIL, or their heteromers expressed as single-chain proteins (*19, 49*). Centotto-1 inhibited APRIL and all heteromers, while Sandy-2 blocked BAFF and to a lesser extent heteromers (Figure 1B) (*48*).

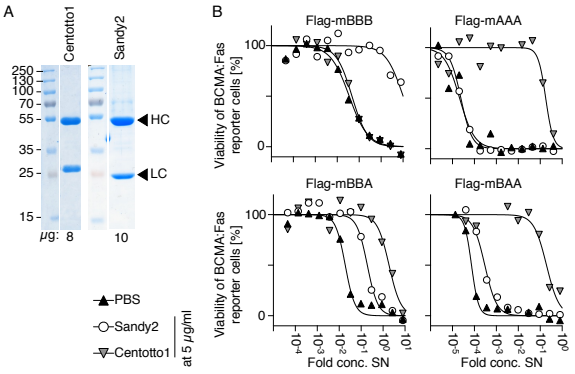


Figure 1. Specificity of anti-APRIL and anti-BAFF neutralizing antibodies. **(A)** Coomassie blue staining of reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 8 µg of anti-APRIL mAb Centotto-1 and 10 µg anti-BAFF mAb Sandy-2. Molecular weight standards were analyzed in the same gels. HC: heavy chain. LC: light chain. **(B)** The indicated Flag-tagged mouse (m) single chain ligands (A, APRIL; B, BAFF) in transfected cell supernatants were titrated on BCMA:Fas reporter cells in the presence of an anti-Flag antibody at a fixed concentration of 1 µg/mL, and plus or minus Centotto-1 or Sandy-2 at a fixed concentration of 5 µg/mL. Reporter cell viability was monitored after an overnight incubation with a colorimetric cell viability assay. This experiment was performed twice with similar results.

BAFF and APRIL are partially redundant to control plasma cells and antibody levels in various compartments.

Next, the effect of neutralizing antibodies was tested in mice. Circulating IgA, IgG, and IgM levels were not significantly modified in naive wild-type (WT) mice by blocking APRIL or BAFF alone but were reduced up to greater than 10-fold by blocking both for 9 wk (Figure 2A), an effect also seen at 3 or 6 wk (Figure S1A). gA and IgG in feces were almost completely abolished after 9 wk of dual treatment, with smaller effects on fecal IgM, while single treatments with anti-APRIL or anti-BAFF only led to a partial reduction (Figure 2B). IgA levels in bronchoalveolar lavage fluids (BALF) were reduced by BAFF inhibition and even more by BAFF/APRIL blockade (Figure S1B). IgG were not reduced in BALF, while IgM in BALF were hardly detectable.

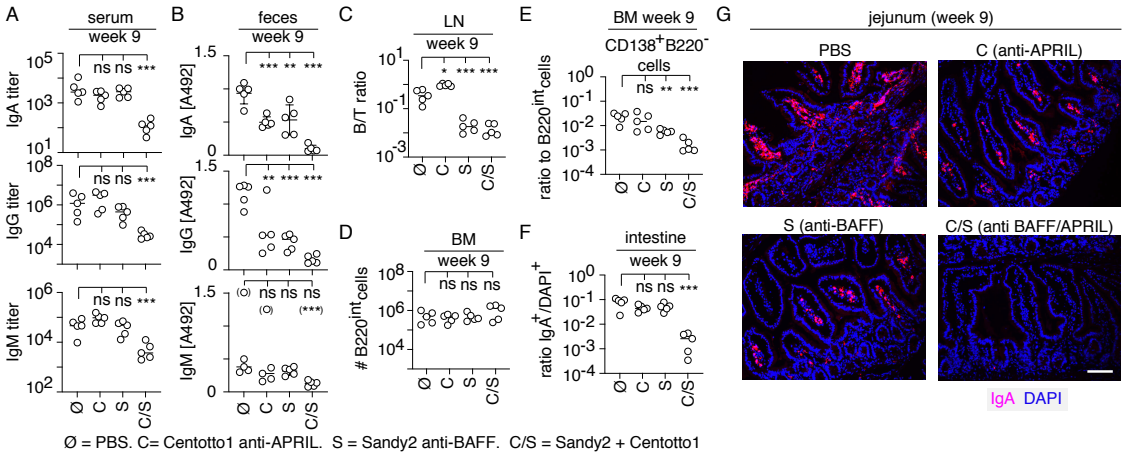


Figure 2. Decrease of plasma cells and antibody levels by inhibition of APRIL and BAFF in WT mice. C57BL/6 mice (n= 5 per group) were treated with PBS (Ø), anti-APRIL Centotto-1 (C), anti-BAFF Sandy-2 (S), or both (C/S) for 9 wk. The analysis was performed at 9 wk. **(A)** Titers of IgA, IgG and IgM measured by ELISA. **(B)** IgA, IgG, and IgM signals measured in feces by ELISA at a single dilution. **(C)** Ratio of B/T cells measured by FACS in lymph nodes (LN). **(D)** Number of immature $B220^{int}$ B cells in the BM (one femur and one tibia). **(E)** Quantification of $CD138^{+}/B220^{-}$ plasma cells in the BM identified by FACS, normalized to immature B cells ($B220^{int}$). **(F)** Quantification of IgA^{+} plasma cells in three pictures/mouse of gut (jejunum) sections stained by immunofluorescence for IgA^{+} cells, normalized to nuclei (DAPI) (ratio of $IgA^{+}/DAPI^{+}$). **(G)** Representative immunofluorescence staining of IgA (red) and DAPI for nuclei (blue) in the jejunum sections of untreated or treated WT mice. Scale bar: 50 µm. Panels A-F: Line at median. One-way ANOVA with Dunnett's multiple comparison test. ns: not significant. * $P < 0.05$. ** $P<0.01$. *** $P<0.001$. The result in parentheses was obtained after exclusion of the indicated outliers. This experiment was performed once. Measures of antibodies in panel A were performed twice with similar results.

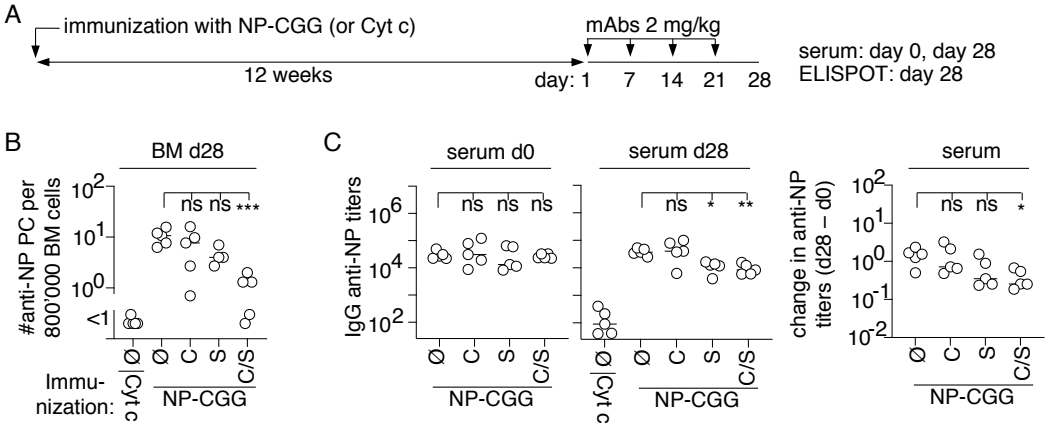
As expected, inhibition of BAFF, but not of APRIL, depleted mature B cells in LN (Figure 2C) and recirculating $B220^{hi}$ mature B cells in the BM, but not the BAFF-insensitive $B220^{int}$ immature BM B cell precursors (Figure 2D, Figure S1C, Figure S2) (*48, 50*). Immature B cells were used to normalize $CD138^{+}$ cells that were separated in populations of dividing plasmablasts ($CD138^{+}/B220^{+}$) versus resting early or mature plasma cells ($CD138^{+}/B220^{-}$) (*51*). These cells are also $TACI^{+}$, but this criterium was not always used, especially not for TACI-deficient cells. BAFF

inhibition decreased plasmablasts and plasma cells in BM, with the double APRIL/BAFF inhibition further reducing plasma cell numbers (Figure 2E, Figure S1D). IgA-positive plasma cells monitored by immunofluorescence (IF) in the jejunum, a portion of the intestine where they are abundant (Figure S1F), were reduced about 10-fold by APRIL/BAFF inhibition, but not by blocking BAFF or APRIL alone (Figure 2F, G). Similar but less pronounced results were also observed for splenic IgA-positive plasma cells (Figure S1E, G). Repeats of this experiment with 4 wk of treatment yielded similar results (Figure S3A-L).

These results indicate that while most mature peripheral B cells depend on BAFF alone, plasma cells are supported by both APRIL and BAFF. Those cells account for the majority of fecal IgA and IgG and for greater than 90% of circulating IgA, IgG, and IgM.

Long-lived T-dependent plasma cells also rely on BAFF and APRIL.

To test the role of BAFF and APRIL for long-lived plasma cells generated in a vaccination setting, mice were injected subcutaneously with a T-dependent antigen (NP-CGG, nitrophenyl-coupled chicken gamma globulin) leading to an adaptative immune response within draining LN. Mice were rested for 12 wk after vaccination and then treated for four additional weeks with antagonists of BAFF and/or APRIL in the absence of an antigenic boost (Figure 3A). After that time, NP-specific plasma cells in the BM were too few for reliable quantification by fluorescence-activated cell sorter (FACS) but could be detected by enzyme-linked immunosorbent spot (ELISPOT) and showed a significant 10-fold reduction upon APRIL/BAFF dual, but not single inhibition (Figure 3B). Mice immunized with an irrelevant antigen (horse cytochrome c) served as negative controls (Figure 3B, C). Reduction in NP-specific plasma cells correlated with a significant 3.6-fold decrease in circulating NP-specific IgG, also seen when considering the difference in titer before and after treatment for each individual mouse (Figure 3C). NP-specific IgA were too low for quantification, while cross-reacting IgM antibodies present also in negative controls prevented NP-specific IgM identification. These results indicate that long-lived IgG^+ plasma cells generated in a T-dependent immunization largely rely on both BAFF and APRIL for their maintenance.



Ø = Isotype control. C= Centotto1 anti-APRIL. S = Sandy2 anti-BAFF.

Figure 3. Depletion of T-dependent, long-lived plasma cells with dual blockade of APRIL and BAFF. (A) Schematic representation of the experiment. C57BL/6 mice were immunized once with nitrophenyl-coupled chicken gamma-globulins (NP-CGG) (or horse cytochrome c as control), rested for 12 wk, then treated for 4 wk with isotype control (Ø), Centotto-1 (C), anti-BAFF Sandy-

2 (S), or both (C/S) (n= 5 mice per group). **(B)** Number of anti-NP$^+$ cells per 800'000 BM cells monitored by ELISPOT. **(C)** Anti-NP IgG titers measured by ELISA in serum at day 0 and after 4 wk (d28) of treatment (left two panels). Difference in anti-NP IgG titers after and before treatment (right panel). Panels B, C: Line at median. One-way ANOVA with Dunnett's multiple comparison test. Ns: not significant. * P < 0.05. ** P<0.01. *** P<0.001. This experiment was performed once.

Depletion of BAFF in APRIL-ko and of APRIL in BAFF-ko mice further decreased Ig levels.

Next, we analyzed antibody levels in untreated APRIL/BAFF double ko mice. Compared with WT mice, they had much lower levels of IgA, IgM, and IgG in the circulation and in feces, less plasma cells in the BM, and less IgA-positive plasma cells in the intestine (Figure 4A-F), reminiscent of our observations with WT mice after dual APRIL/BAFF inhibition (Figure 2). Even though WT and APRIL-ko cohorts were not analyzed side by side, APRIL-ko mice resembled WT mice apart for lower IgA levels in serum and in feces (Figure 4A, F). Inhibition of BAFF in APRIL-ko animals further reduced IgA levels in serum and BALF, gut IgA plasma cells, and except for one outlier result, IgA levels in feces, indicating that BAFF alone supported a fraction of the IgA response (Figure 4A-F, Figure S4). Similarly, inhibition of APRIL in BAFF-ko, which have lower B cells than WT, reduced IgA levels in serum, feces, and decreased IgA$^+$ plasma cells in the gut (Figure 4A-C, F). In BAFF-ko mice, inhibition of APRIL did not further reduce the proportion of mature B cells in LN (Figure 4C), but further decreased BM plasma cells (Figure 4E).

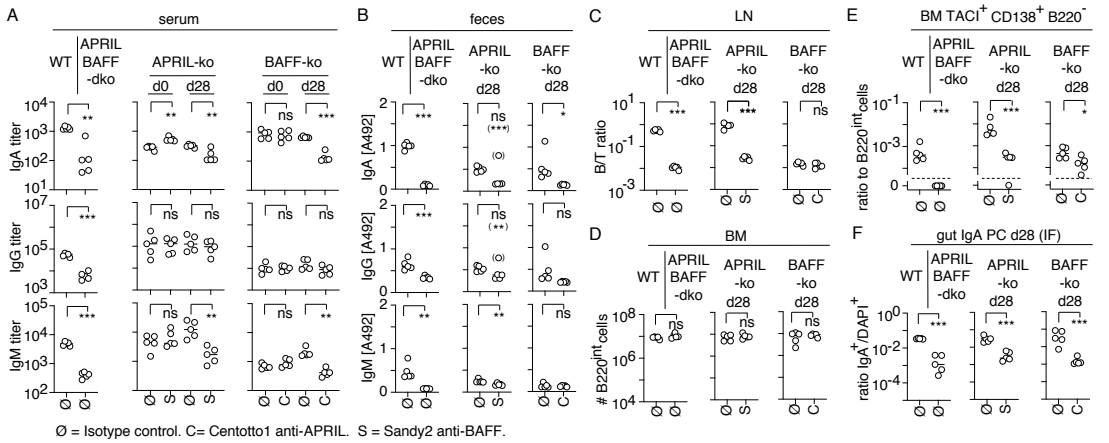


Fig. 4. Inhibition of APRIL and BAFF in ligand-deficient mice confirms roles for both APRIL and BAFF to maintain plasma cells. BAFF-ko and APRIL-ko mice were treated with isotype control (Ø), anti-APRIL Centotto-1 (C), or anti-BAFF Sandy-2 (S) for 4 wk. Isotype control-treated WT and APRIL/BAFF double ko mice were also analyzed (n= 5 per group). APRIL-ko, BAFF-ko, and WT plus double ko were analyzed in independent cohorts and are not necessarily directly comparable. **(A)** Titers of IgA, IgG, and IgM in serum before (d0) and after 4 wk (d28) of treatment. **(B)** IgA, IgG, and IgM signals measured by ELISA in feces at 4 wk at a single dilution. **(C)** Ratio of B/T cells measured by FACS in lymph nodes (LN). **(D)** Number of immature B220^{int} B cells in the BM measured by FACS. **(E)** Quantification of CD138$^+$/B220$^-$/TACI$^+$ plasma cells identified by FACS, normalized to immature B cells (B220^{int}). **(F)** Quantification of IgA$^+$ plasma cells in three pictures/mouse of gut (jejunum) sections stained by immunofluorescence (IF) for IgA, normalized to DAPI for nuclei (ratio of IgA$^+$/DAPI$^+$). Lines indicate median. One-way ANOVA with Dunnett's multiple comparison test. Ns: not significant. * P < 0.05. ** P<0.01. *** P<0.001. The result in parentheses was obtained after exclusion of the indicated outlier (possibly caused by some blood

in stool). This experiment was performed once. Measures of antibodies in panel A were performed twice with similar results.

In conclusion, the inhibitory activities of anti-APRIL and anti-BAFF antibodies were validated and
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phenotypes of BAFF and APRIL inhibition mirrored those of ko animals, with the notable exception of circulating IgG levels that were reduced in APRIL/BAFF double ko mice, but not in APRIL-ko mice treated with the anti-BAFF antibody (Figure 4A). These results also demonstrate that in the absence of BAFF, APRIL can maintain at least a fraction of IgA plasma cells, and IgA and IgM antibody levels in various fluids.

Depletion of BAFF and APRIL in TACI-ko, BCMA-ko, and TACI/BCMA double ko mice reveal three physiologic ligand - receptor pairs for maintenance of plasma cells.

To identify the relevant ligand–receptor pairs which support plasma cells and thereby antibody levels, APRIL and/or BAFF were blocked for 4 wk in mice expressing either BAFFR alone (TACI/BCMA double ko), or BAFFR and TACI only (BCMA-ko), or BAFFR and BCMA only (TACI-ko). Relative to WT mice, TACI/BCMA double ko mice displayed lower levels of IgA and IgM in serum, feces, and BALF, lower levels of plasma cells in the BM, and lower levels of gut IgA^+ plasma cells, usually by a factor of about 10. These defects were not further enhanced by pharmacological inhibition of BAFF and APRIL, indicating that residual plasma cells and antibody levels do not depend on BAFF and APRIL, and excluding a major contribution of BAFF–BAFFR interactions in plasma cell maintenance (Figure 5 and figure S5). In TACI-ko mice (having BCMA and BAFFR), parameters related to IgA and IgM, including BM plasma cells, responded equally well to the inhibitions of APRIL or APRIL/BAFF, but were unaffected by the inhibition of BAFF alone, indicating that the contribution of BCMA to plasma cell maintenance depends exclusively on APRIL (Figure 5 and figure S5). In contrast, in BCMA-ko mice (having TACI and BAFFR), all parameters related to IgA and IgM responded strongly to APRIL/BAFF inhibition, and sometimes moderately to the inhibition of BAFF alone, but never to the inhibition of APRIL alone, indicating that TACI responded to either APRIL or BAFF, with an overall larger contribution of BAFF.

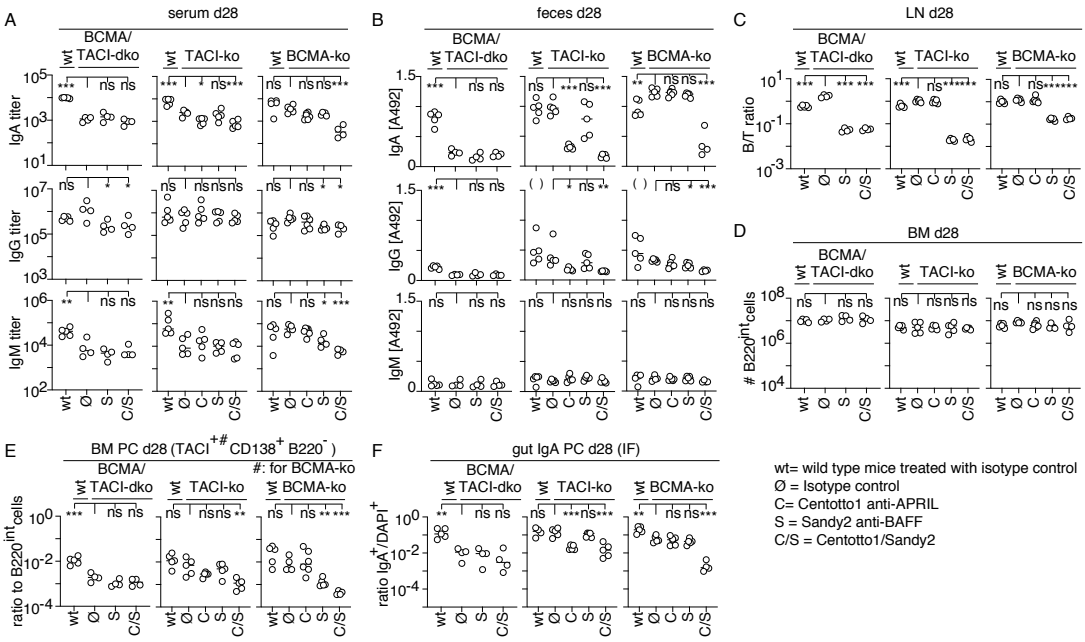

Figure 5. Inhibition of APRIL and BAFF in receptor-deficient mice reveals three functional ligand – receptor pairs for maintenance of plasma cells and antibodies. BCMA-ko, TACI-ko, and BCMA/TACI double ko mice were treated with isotype control (Ø), anti-APRIL Centotto-1 (C) anti-BAFF Sandy-2 (S) or both (C/S) for 4 wk (n= 5 per group, except double ko n = 4 per group). BCMA-ko, APRIL-ko, and double ko mice were analyzed in independent cohorts and are not necessarily directly comparable, but all groups contained a control group of wild type mice (n = 5) treated with the isotype control. **(A)** IgA, IgG, and IgM titers measured by ELISA in serum after 4 wk of treatment (d28). **(B)** IgA, IgG, and IgM signals measured by ELISA at a single dilution in feces at 4 wk. **(C)** Ratio of B/T cells measured by FACS in lymph nodes (LN). **(D)** Number of immature $B220^{int}$ B cells in the BM. **(E)** Quantification of $CD138^{+}/B220^{-}$ (or $CD138^{+}/B220^{-}/TACI^{+}$ for BCMA-ko) plasma cells identified by FACS, normalized to immature B cells ($B220^{int}$). **(F)** Quantification of IgA^{+} plasma cells in three pictures/mouse of gut (jejunum) sections stained by immunofluorescence (IF) for IgA, normalized to DAPI for nuclei (ratio of $IgA^{+}/DAPI^{+}$). Lines indicate median. One-way ANOVA with Dunnett's multiple comparison test. Ns: not significant. * $P < 0.05$. ** $P<0.01$. *** $P<0.001$. This experiment was performed once. Measures of antibodies in panel A were performed twice with similar results.

Together, these results show that the BAFF-BAFFR axis well known to support survival of mature B cells (Figure 5C) has no or only negligible impact on plasma cells that instead use BAFF-TACI, APRIL-TACI and APRIL-BCMA interactions for their maintenance in a physiological context, when IgA or IgM levels were measured as end points.

If plasma cells use only BCMA and TACI, but not BAFFR to survive, IgG levels should be constitutively decreased in TACI/BCMA double ko mice, which was not the case (Figure 5A). Yet, a specific BAFF-BAFFR survival axis for IgG plasma cells is unlikely because IgG levels in BCMA/TACI double ko did not or only marginally respond to BAFF inhibition (Figure 5A). This raises the question of whether IgG plasma cells are intrinsically different from IgA or IgM ones in

their response to BAFF, and/or whether compensatory mechanisms specific for IgG plasma cells were up-regulated in BCMA/TACI double ko mice, and/or whether the experimental model based on a 4 wk-long ligand inhibition was inadequate to detect meaningful changes in circulating IgG levels.

BAFF and APRIL contribute to maintaining IgG, IgA, and IgM plasma cells in the BM and spleen; splenic IgG plasma cells also require IL-6.

IgGs have a longer half-life in blood due to interactions with neonatal Fc receptor (FcRn) (*52*). This specifically delays the decrease of circulating IgGs even if all plasma cells are depleted. This parameter was minimized by treating FcRn (*fcgrt*)-deficient mice for 4 wk with combinations of anti-APRIL/BAFF. Alternatively, FcRn-deficient mice were also treated with anti-IL-6 antibodies, given that IL-6 deficient mice have also been associated with lower intestinal IgA^+ plasma cell numbers (*53, 54*). Anti-APRIL/BAFF decreased IgA in serum, BALF, and feces, but anti-IL-6 did not whether administered alone or in combination with anti-APRIL/BAFF (Figure 6A, B). IgG levels in serum and feces were reduced by both anti-APRIL/BAFF or anti-IL-6 treatments, with a possible additional effect of the anti-APRIL/BAFF/IL-6 combination. Similar results were obtained for IgM levels in serum and feces, except that the effect of anti-IL-6 was best seen in combination with anti-APRIL/BAFF (Figure 6A, B, Table 1). Successful inhibition of BAFF was also witnessed by the reduction of mature B cells in LN (Figure 6C). $CD138^+$ antibody-producing cells in the BM and spleen were normalized to immature B cells and $CD3^+$ T cells, respectively, whose numbers were insensitive to BAFF/APRIL/IL-6 blockade (Figure 6D), and further subdivided into $B220^+/CD19^+$ (plasmablasts), $B220^-/CD19^+$ (early plasma cells, or short-lived), and $B220^-/CD19^-$ (mature plasma cells, or long-lived) (Figure 6E) with additional staining for intracellular IgG, IgA, or IgM. Relatively minor populations of triple-negative or IgA plus IgM double-positive cells were detected but not further analyzed. In the BM, the anti-APRIL/BAFF treatment decreased early and mature plasma cells of all isotypes, and decreased IgA^+, but not IgG^+ or IgM^+ plasmablasts, while anti-IL-6 had no detectable effect alone or added to anti-APRIL/BAFF (Figure 6F). The situation was distinct in the spleen: Anti-IL-6 alone selectively decreased IgG^+ plasmablasts and plasma cells, not IgA^+ and IgM^+ ones, while anti-APRIL/BAFF reduced all plasmablasts and plasma cells except IgG^+ plasma cells (Figure 6G). For splenic IgG^+ plasmablasts, the effect of anti-IL-6 and anti-APRIL/BAFF was cumulative (Figure 6G). Similarly, anti-IL-6 may potentiate the inhibition of long-lived IgM plasma cells deprived of APRIL and BAFF, and anti-APRIL/BAFF may potentiate the inhibition of long-lived IgG^+ plasma cells deprived of IL-6 (Figure 6G). IgA^+ plasma cells detected by immunohistochemistry in the gut and spleen were dependent on APRIL/BAFF, but not on IL-6 (Figure 6H, I and figure S6).

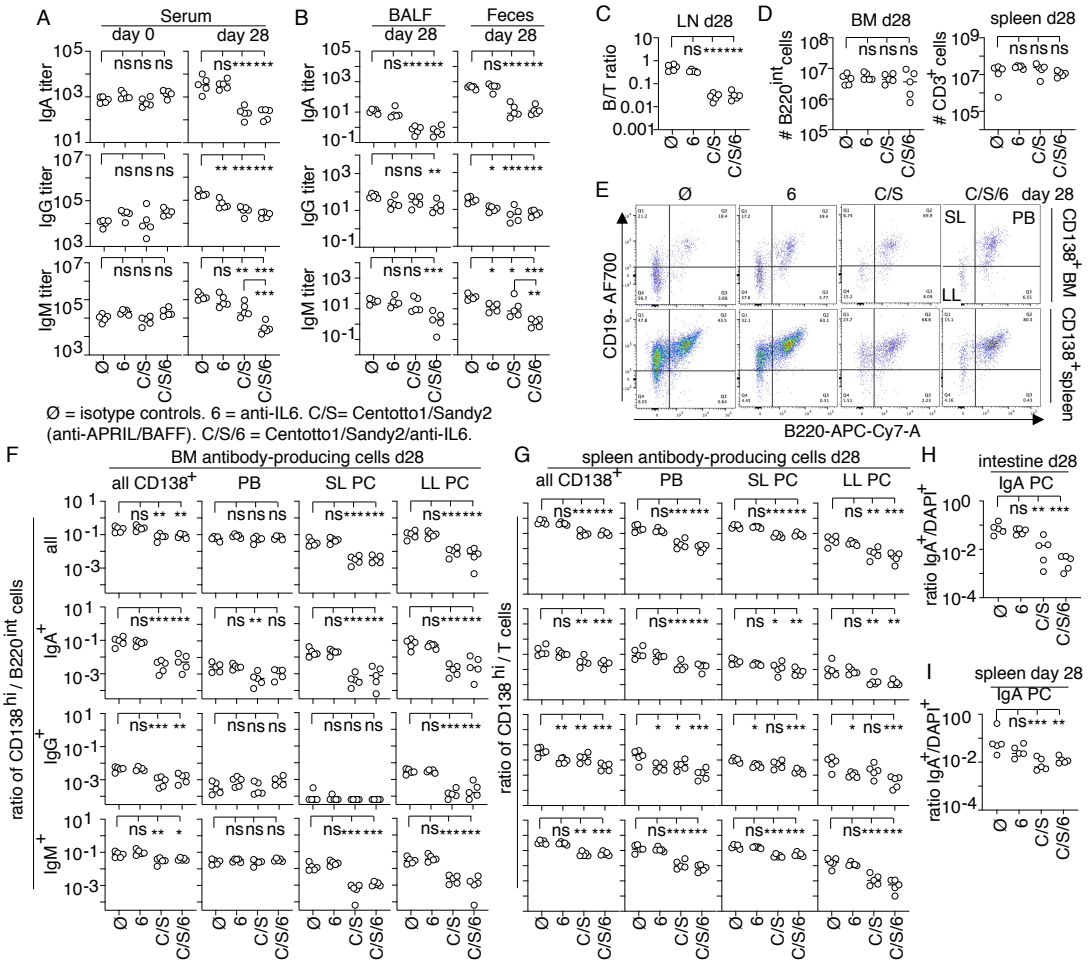

Fig. 6. Inhibition of APRIL/BAFF and/or IL-6 in FcRn (*fcgrt*)-deficient mice indicates complementary functions of distinct factors for plasma cell maintenance. FcRn (*fcgrt*)-deficient mice were treated with isotype controls (Ø), anti-IL-6 (6), anti-APRIL/anti-BAFF Centotto-1/Sandy-2 (C/S), or all three inhibitory antibodies (C/S/6) for 4 wk (n= 5 per group). **(A)** IgA, IgG, and IgM titers measured by ELISA in serum before (d0) or after 4 wk (d28) of treatment. **(B)** IgA, IgG, and IgM signals measured by ELISA at a single dilution in bronchoalveolar fluids or in feces at 4 wk. **(C)** Ratio of B/T cells measured by FACS in lymph nodes (LN). **(D)** Number of immature $B220^{int}$ B cells in the BM, and of $CD3^+$ T cells in spleen at 4 wk. **(E)** Identification of $CD138^+/B220^+/CD19^+$ plasmablasts (PB), $CD138^+/B220^-/CD19^+$ early (short-lived) plasma cells (SL) and $CD138^+/B220^-/CD19^-$ mature (long-lived) plasma cells (LL) within $CD138^+$ antibody-producing cells in the BM and spleen. **(F)** Numbers of IgA^+, IgG^+ and IgM^+ plasmablasts (PB), short-lived and long-lived plasma cells (SL-PC, LL-PC) normalized to $B220^{int}$ immature B cells in the BM after 4 wk of treatment. **(G)** Same as panel F, except that splenic cells were analyzed and normalized to $CD3^+$ T cells. **(H)** Quantification of IgA^+ plasma cells in three pictures/mouse of intestine (jejunum) sections and **(I)** in spleen section stained by immunofluorescence for IgA, normalized to DAPI for nuclei (ratio of $IgA^+/DAPI^+$). Panels A-D and F-I: Lines indicate median. One-way ANOVA with Dunnett's multiple comparison test. Ns: not significant. * $P < 0.05$. ** $P<0.01$. *** $P<0.001$. This experiment was performed once.

Table 1. Percentage of average decrease of IgA, IgG, and IgM levels relative to control treatment in plasma, broncho-alveolar lavage fluids (BALF) and feces of FcRn-deficient mice treated for 4 wk with anti-IL-6, anti-APRIL/BAFF (Centotto-1 + Sandy-2) or anti-APRIL/BAFF/IL-6 (Centotto-1 + Sandy-2 + anti-IL-6).

Sample	Isotype (time)	Treatment: anti-IL-6	Treatment: Centotto-1 + Sandy-2	Treatment: Centotto-1 + Sandy-2 + anti-IL-6
Plasma	IgA (week 0)	< 30	< 30	< 30
Plasma	IgA (week 4)	< 30	94.1	94.8
BALF	IgA (week 4)	32.5	94.2	95.3
Feces	IgA (week 4)	< 30	97.2	97.1
Plasma	IgG (week 0)	< 30	< 30	< 30
Plasma	IgG (week 4)	64.7	81.1	87.6
BALF	IgG (week 4)	59.7	51.5	74
Feces	IgG (week 4)	67.4	84.5	81.2
Plasma	IgM (week 0)	< 30	< 30	< 30
Plasma	IgM (week 4)	41.8	81.4	98
BALF	IgM (week 4)	< 30	42.9	94.9
Feces	IgM (week 4)	79.5	81.9	97.9

These results unveil complementary roles of IL-6 and of APRIL and BAFF for plasma cell maintenance. IL-6 supports IgG^+ plasmablasts and plasma cells in the spleen, correlating with decreased IgG in serum and in feces. APRIL and BAFF support all IgA^+ cells examined and decreased IgA levels in serum, BALF, and feces. APRIL and BAFF also support IgG^+ and IgM^+ producing cells, except plasmablasts in the BM, correlating in FcRn-ko mice with decreased IgG and IgM levels in serum and feces.

Discussion

The involvement of BAFF and APRIL on the survival of plasma cells has previously been reported (*12, 55, 56*), but the question of possible compensatory mechanisms when using knock-out mice was left unanswered. Also, none of these studies disentangled contributions of APRIL, BAFF, and their heteromers binding to BAFFR, BCMA, TACI, and proteoglycans. We addressed these questions with several knock-out mice and two function-blocking antibodies against APRIL

(Centotto-1) or BAFF (Sandy-2) that can phenocopy genetic deficiencies of their targets. Both antibodies are monoclonal mouse IgG1, reducing the risk of neutralizing anti-drug antibody responses that have been observed previously in autoimmune mice treated with Apry 1-1, a human single-chain anti-mouse APRIL antibody (*55, 56*).

We find that early and mature plasma cells ($CD138^+/B220^-$) in the BM of WT mice can thrive on either APRIL or BAFF, but not in the absence of both. In contrast, $CD138^+/B220^+$ plasmablasts resist better the loss of APRIL and BAFF and could contribute to residual Ig levels post pharmacologic or genetic inhibition.

APRIL/BAFF do not have the monopole of antibody-secreting cell survival: Splenic IgG^+ plasma cells depended on IL-6 much more than on APRIL/BAFF, which could in part explain why effects of APRIL and/or BAFF inhibition were often better visible on IgA or IgM than on IgG parameters. We speculate that upregulation of IL-6, IL-6R, or other survival factor for IgG^+ plasma cells may compensate for loss of BAFF and APRIL signaling in plasma cells of the BCMA/TACI double ko mice that had no decrease in IgG levels.

In WT mice, except for BM plasmablasts and splenic IgG^+ plasma cells, the BAFF/APRIL axis was required and nonredundant for about 90% of antibody-secreting cells examined in this study, including preformed and long-lived T-dependent ones, and accounted for about 90% of the total antibody production.

This study also demonstrated the exclusive response of BCMA to APRIL, and the nonexclusive response of TACI to both BAFF and APRIL. The paucity of mature plasma cells observed in the gut and BM of TACI/BCMA double ko mice argues against an important role of BAFFR in these cells, a conclusion reinforced by lack of further amplification of the phenotype of TACI/BCMA double ko mice with anti-BAFF/APRIL. This same result argues against an important self-sufficient role of APRIL binding to proteoglycans in plasma cell maintenance, in as much as anti-APRIL antibody would interfere with this interaction [but does not preclude a role of proteoglycans to retain APRIL in the vicinity of, or to present APRIL to TACI and/or BCMA (*57, 58*)). Also, the reduction of IgA parameters in BAFF-ko mice treated with anti-APRIL, or in APRIL-ko mice treated with anti-BAFF, demonstrated that “pure” APRIL and pure BAFF can both support plasma cells in vivo, excluding unique contributions of heteromers in this function. It is noteworthy that the rare humans identified with homozygous deficiencies in either BAFFR (*59*) or APRIL (*60*) had common variable immunodeficiency, which is characterized by low antibody levels, confirming that both BAFF and APRIL participate to antibody production in humans in a clinically relevant manner.
The dependence of the IgA response on both BAFF and APRIL, but not IL-6, could be explained by the death of preformed IgA plasma cells in the absence of BAFF/APRIL or by impaired IgA class-switch that can be induced by BAFF, APRIL, or both (*30*). Class-switch inhibition is unlikely the sole cause of IgA titer decrease because no concomitant increase of serum IgM titers or IgM^+ plasma cells was observed.

Therapeutic regulation of IgA levels is of clinical interest because pathological deposition of secretory, galactose-deficient IgA in the kidney can cause IgA nephropathy and proteinuria (*46*). BAFF-transgenic mice overexpressing BAFF display an enormous IgA production in response to commensal bacteria, leading to kidney damage, but this is normalized under germfree conditions (*61*); the approved anti-human BAFF antibody belimumab reduced proteinuria in lupus nephritis

(*62*); in a mouse model of IgA nephritis, kidney parameters were improved by blocking APRIL (*63*); a human anti-human APRIL antibody decreased IgA levels in non-human primates (*63*) and soluble TACI-based drug that inhibits both BAFF and APRIL significantly reduced IgA levels and proteinuria in patients with IgA nephropathy (*64*). Our findings indicate that IgA plasma cells, and especially those in the gut mucosa are supported by all three BAFF-TACI, APRIL-TACI and APRIL-BCMA ligand-receptor pairs under normal conditions and that the BAFF/APRIL system is not redundant (at least not with IL-6) for the vast majority of IgA plasma cells. This reinforces the rational of these different therapeutic trials and predicts that dual inhibition of BAFF and APRIL might be the most efficient strategy to reduce plasma cells, perhaps at the expense of stronger impacts on the normal immune function.

Materials and Methods

Animals.

C57BL/6JOlaHsd mice were purchased from Envigo. BAFF-ko (*50*), APRIL-ko (*65*), TACI-ko, BCMA-ko and TACI/BCMA double ko (*25*) mice were as previously described. B6.129-*Fcgrt*^{tm1Dcr}/DcrJ mice (003982) were obtained from Jackson Laboratory. Mice were handled under authorizations VD1370.7, VD1370.8 and VD1370.9 of the veterinarian service of canton de Vaud and according to Swiss Federal Office guidelines. The genotype of all mice used in experiments was confirmed by PCR.

Cells and reagents.

HEK293T cells were grown in Dulbecco's modified Eagle medium (DMEM) 10% fetal calf serum. Jurkat JOM2-BCMA:Fas-2309cl13 were as described (*49*). Single-chain Flag-mBBB, Flag-mBBA, Flag-mBAA, and Flag-mAAA (m: mouse, B: BAFF, A: APRIL) were produced by transient transfection of HEK293T cells with polyethylenimide (*67*), as described in supplemental methods. Plasmids used in this study are described in Table S1. Mouse IgG1 anti-mAPRIL blocking monoclonal Centotto-1 (AG-20B-0083) (*47*) and mouse IgG1 anti-mBAFF blocking monoclonal antibody Sandy-2 (AG-20B-0063PF) (*48*) are available from Adipogen. Centotto-1 and Sandy-2 were analyzed by reducing SDS-PAGE and Coomassie blue staining using a semidry iD Stain System (Eurogentech). Mouse IgG1 anti-EDA1 nonblocking monoclonal antibody EctoD1 was as described (*66*). Rat IgG1 anti-mouse IL-6, clone MP5-20F3 was from BioXCell. Rat IgG1 anti-hTRAILR2, clone 4H6.20, which does not cross-react with mouse TRAILR2, was produced in house. Endotoxin levels of antibodies were <0.01 EU/µg.

Treatment and immunization of mice.

Ten- to fourteen-week-old adult WT C57BL/6J, BAFF-ko, APRIL-ko, TACI-ko, BCMA-ko, TACI/BCMA double ko, or FcRn-ko mice were treated weekly with i.p. administration of isotype control(s), anti-APRIL (Centotto-1), anti-BAFF (Sandy-2), anti-IL-6, or combinations thereof, each at 2 mg/kg, for 4 to 9 wk as indicated in figure legends. For single treatments or controls, omitted antibodies were replaced by isotype control(s). In one experiment, phosphate-buffered saline (PBS) was used instead of isotype controls. One week after the last injection, mice were killed by i.p. injection of pentobarbital at 150 mg/kg.

Ten-week-old adult WT C57BL/6J mice were immunized i.p. with 100 µg of NP-CGG (Biosearch Technologies, N-5055E-5) or 100 µg of cytochrome c (C2506, Sigma) mixed with 100 µL of Imject

Alum adjuvant (Thermo Fisher, 77161) in a final volume of 200 µL. Twelve weeks after immunization, mice were treated with 4 weekly injections of isotype control, anti-APRIL, anti-BAFF, or anti-APRIL/BAFF at 2 mg/kg. Mice were analyzed 1 wk after the last injection.

Blood samples were collected before administration of the antibodies and the day before the mice were killed by puncture of the facial vein (*68*). Blood samples were clotted for 2 h at 37 °C, after which time they were spun at 13,300 rpm (17,000 × g) for 10 min at 4 °C. Serum was stored at −20 °C until use. After the mice were killed, they were tracheotomized, and BALF were collected by washing lungs with 500 µL of PBS and 10 µg/mL bovine serum albumin (BSA) using dedicated catheters (Troge, 18G green, 45/32 length). BALF was centrifuged for 10 min at 4 °C, and the supernatant was stored at −20 °C. Fecal samples from the colon were collected in preweighed tubes, lyophilized, weighed again, and dissolved at 20 mg/mL in PBS 0.05% azide. Samples were spun at 13,300 rpm (17,000 × g) for 10 min at 4 °C, and supernatants were stored at −20 °C until use.

Cytotoxicity assay.

Jurkat BCMA:Fas cl13 reporter cells in Roswell Park Memorial Institute medium (RPMI) 10% fetal calf serum were incubated with titrated amounts of single-chain Flag-tagged mouse BAFF, APRIL, or heteromers in conditioned cell supernatants in the presence of anti-Flag-M2 antibody (Sigma, #F3165) at a final concentration of 1 µg/mL and of anti-mouse APRIL Centotto-1 or anti-mouse BAFF Sandy-2 at a final concentration of 5 µg/mL for 16 h at 37 °C, 5% CO_2. Then, 20 µL of PMS/MTS (phenazine methosulfate at 0.9 mg/mL in PBS (PMS)/[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium at 2 mg/mL in PBS (MTS) (1:20, v/v)] was added for further incubation of 6 to 8 h before absorbance reading at 492 nm.

Enzyme-linked immunosorbent assay (ELISA)

Titers of IgA, IgM, and IgG were measured by the sandwich ELISA as described in detail in supplemental method using, respectively, i) goat anti-mouse IgA/biotinylated anti-mouse IgA, ii) goat anti-mouse IgM µ chain-specific/biotinylated anti-mouse IgM, and iii) goat anti-mouse IgG γ chain-specific/biotinylated anti-mouse IgG (H + L). NP-specific antibody isotypes were measured by coating NP-25 BSA (Biosearch Technologies, #N5050-100) and revealing with biotinylated anti-IgA, -IgG, or -IgM. Where indicated, measures were performed at a single sample dilution (BALF 1/1, 1/25, and 1/5 for IgA, IgG, and IgM; feces at 1/20 for IgA and 1/100 for IgG and IgM).

Flow cytometry.

The identification of lymphocytes and plasma cells in spleen, LN, and BM samples by flow cytometry was performed as described in supplemental methods, Tables S2 and S3 and Figure S1. Briefly, anti-B220, -CD3, -CD138, -CD19, and -TACI antibodies were used to identify B and T cells, plasmablasts, and plasma cells. In one experiment, isotypes produced by plasma cells were identified by intracellular staining with anti-IgA, anti-IgG, and anti-IgM.

Immunofluorescence.

Sections (6 to 8 µm thick) of the spleen and intestine embedded in optimal cutting temperature (OCT) compound were stained with goat anti-mouse IgA (Novus, #NB7501) and counterstained with DAPI. Slides were mounted, imaged, and analyzed to monitor the ratio of IgA-positive cells to nuclei as described in supplemental methods.

ELISPOT.

ELISPOT was performed as described in supplemental method. Briefly, filter plates coated with NP-25-BSA (Biosearch, #N-5050-100) were blocked with DMEM, 10% fetal calf serum. A total of 800,000 BM cells/well were added in triplicate and left overnight at 37 °C, 5% CO_2. Spots were revealed with goat anti-mouse IgG (H + L) (Jackson ImmunoResearch #115-035-146) followed by aminoethylcarbazole (AEC)/H_2O_2 solution.

Statistical analyses.

Normal distribution of samples was assessed with Shapiro–Wilk and Kolmogorov–Smirnov normality tests. Comparison of multiple groups was performed by ordinary one-way ANOVA followed, if significant, by Dunnett's multiple comparison tests comparing the mean of sample tests to the mean of a control. Comparison of two groups was performed with paired Student's *t* test using Prism software (GraphPad Software, version 7). Differences were considered statistically significant when $P < 0.05$.

Data, materials, and software availability.

Supporting data are available in the Zenodo repository, DOI: 10.5281/zenodo.12562309. All other data are included in the article and/or supplemental method.

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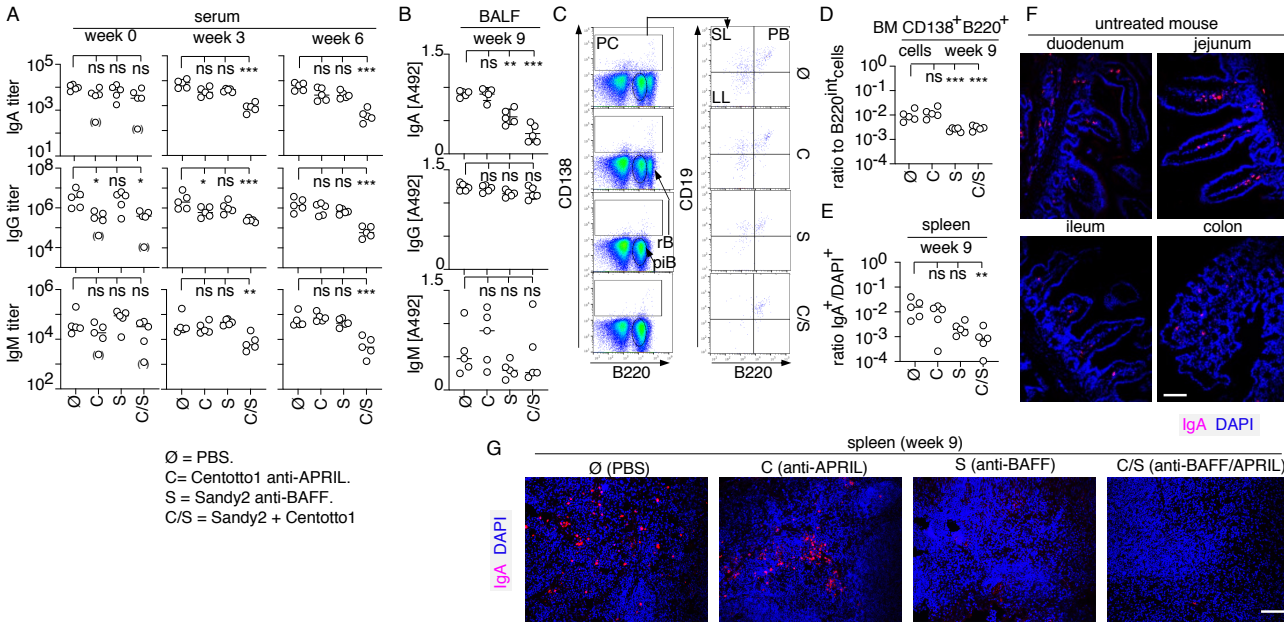
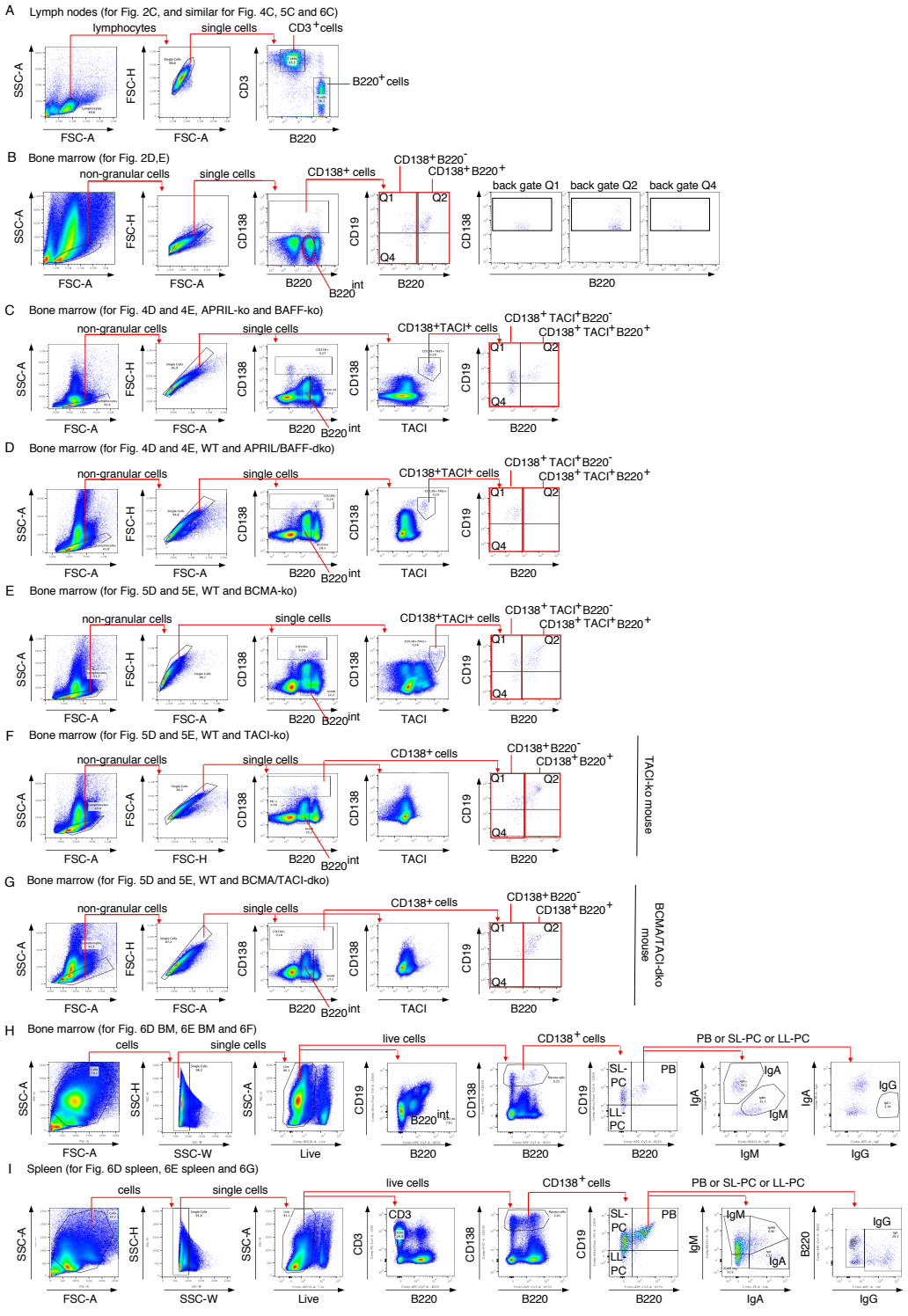
Supplementary Material

Supplementary Figures for Eslami et al.

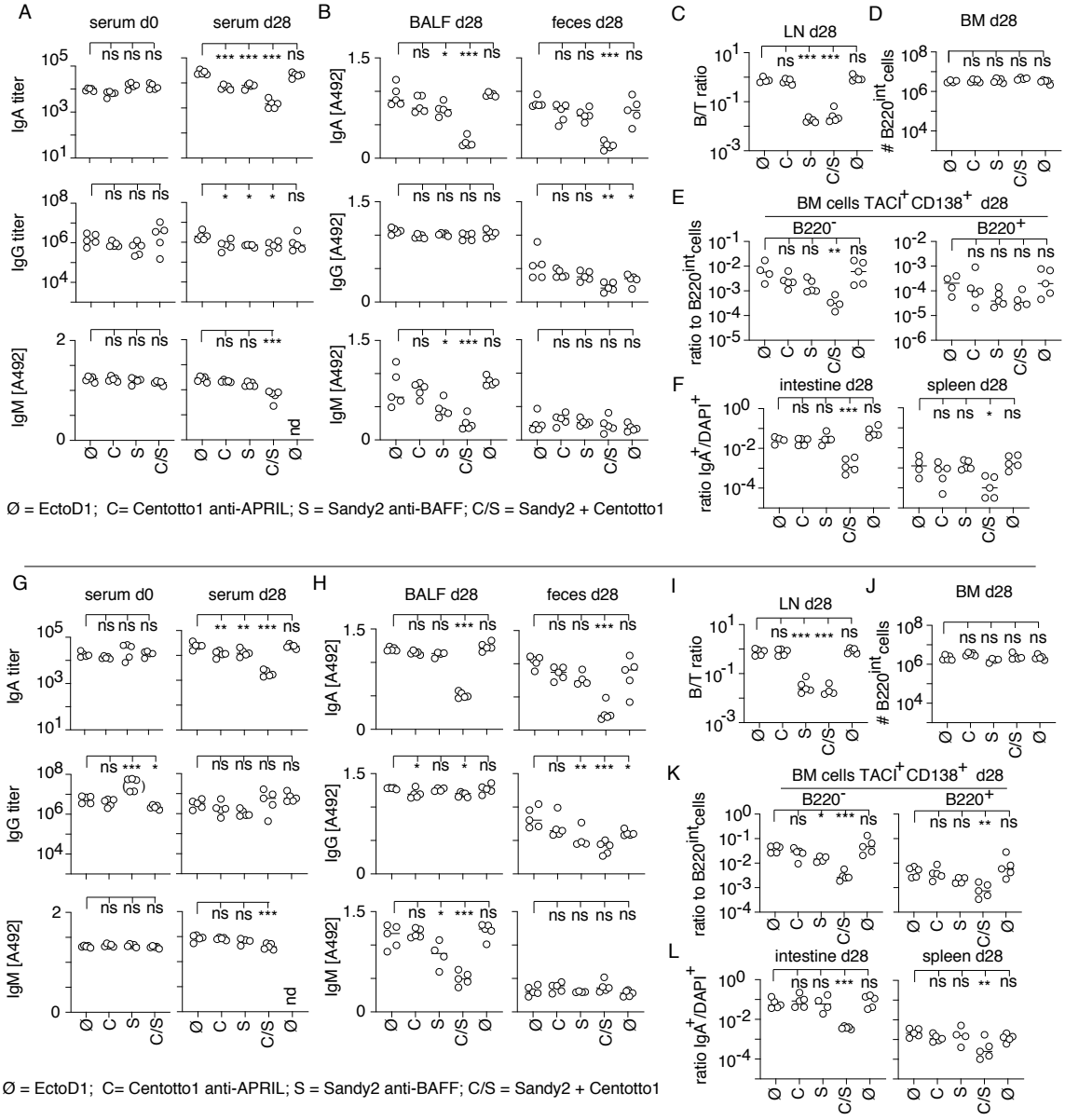


Supplementary figure S1 | Related to Figure 2. Inhibition of APRIL and BAFF in WT mice. C57BL/6 mice were treated with PBS (Ø), anti-APRIL Centotto-1 (C), anti-BAFF Sandy-2 (S), or both (C/S) for 9 weeks (n= 5 per group). **(A)** Titers of IgA, IgG and IgM measured by ELISA in serum collected before treatment (week 0) or after 3 or 6 weeks of treatment. **(B)** IgA, IgG, and IgM signals measured by ELISA at a single dilution (1:1, 1:25, 1:5, respectively) of bronchoalveolar fluids (BALF) at 9 weeks. **(C)** FACS scattergrams of bone marrow cells at 9 weeks in the lymphocyte gate. $CD138^+$ plasma cells (PC) were separated into $B220^+$ plasmablasts (PB) and $B220^-$ short-lived ($CD19^+$, SL) and long-lived ($CD19^-$, LL) plasma cells. piB: $B220^{int}$ pre B and immature B cells. rB: $B220^{high}$ recirculating B cells. **(D)** Quantification of $CD138^+/B220^+$ plasmablasts identified by FACS, normalized to immature B cells. **(E)** Quantification of IgA^+ plasma cells in spleen sections stained by immunofluorescence for IgA and by DAPI for nuclei (ratio of $IgA^+/DAPI^+$). **(F)** Immunofluorescence staining for IgA (red), with DAPI staining of nuclei (blue) in the indicated intestinal portions of the gut of an untreated mouse. Scale bar: 50 μm. **(G)** Immunofluorescence staining as described in panel F but for spleen sections of mice with 9 weeks of the indicated treatments. Scale bar: 50 μm. Panels A, B, D, E: Line at median. One-way ANOVA with Dunnett's multiple comparison test. Ns: not significant. * $P < 0.05$. ** $P<0.01$. *** $P<0.001$.



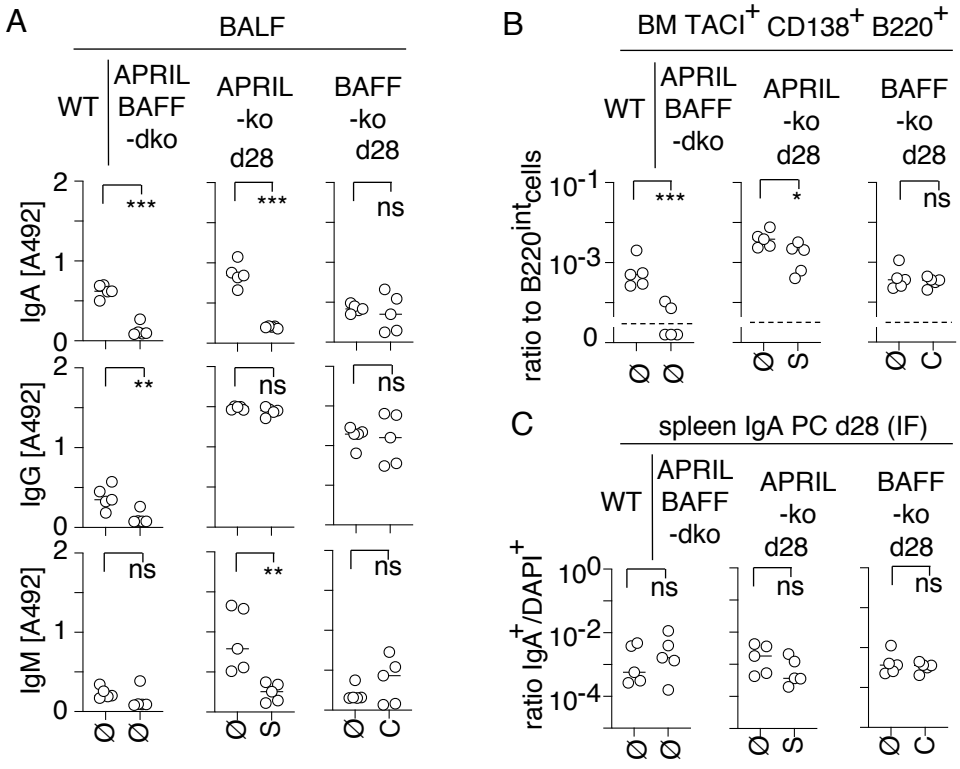
Supplementary figure S2 | Related to Figures 2, 4, 5 and 6. Gating strategies. **(A)** Gating strategy to identify B cells ($B220^+$) and T cells ($CD3^+$) in lymph nodes. **(B)** Gating strategy to identify immature B cells ($B220^{int}$) and plasma cells ($CD138^+/B220^+$ and $CD138^+/B220^-$) in the bone marrow of wild type mice for Fig. 2. The last three panels show back gating of population in quadrants Q1, Q2 and Q4. **(C-G)** Gating strategy to identify immature B cells ($B220^{int}$) and plasma cells ($CD138^+/TACI^+/B220^+$ and $CD138^+/TACI^+/B220^-$; or $CD138^+/B220^+$ and

CD138$^+$/B220$^-$) in the bone marrow of mice of the indicated genotypes for Fig. 4 and 5. Examples shown are for a wild type mouse, except in panel F that shows a TACI-ko mouse and in panel G that shows a BCMA/TACI double ko mouse. **(H)** Gating strategy to identify immature B cells (B220^{int}) and IgA$^+$, IgG$^+$, IgM$^+$ plasmablasts (PB: CD138$^+$/B220$^+$), short-lived plasma cells (SL-PC: CD138$^+$/B220$^-$/CD19$^+$) and long-lived plasma cells (LL-PC: CD138$^+$/B220$^-$/CD19$^-$) in the bone marrow of FcRn-deficient mice from Fig. 6. **(I)** Gating strategy to identify T cells (CD3$^+$) and IgA$^+$, IgG$^+$, IgM$^+$ plasmablasts (PB: CD138$^+$/B220$^+$), short-lived plasma cells (SL-PC: CD138$^+$/B220$^-$/CD19$^+$) and long-lived plasma cells (LL-PC: CD138$^+$/B220$^-$/CD19$^-$) in the spleen of FcRn-deficient mice from Fig. 6.



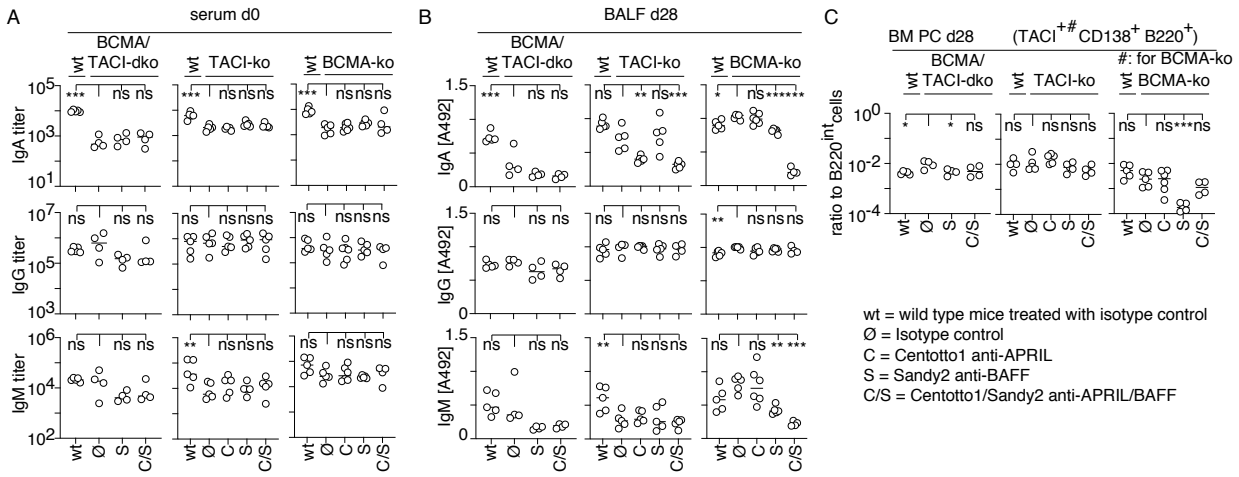
Supplementary figure S3 | Related to Figures 2 and 3. Inhibition of APRIL and BAFF for 4 weeks in WT mice. 10-week old C57BL/6 mice were immunized once with nitrophenyl-coupled chicken gamma-globulins (NP-CGG) (or horse cytochrome c as control), rested for 2 weeks (panels A to F) or 12 weeks (panels G to L), then treated for 4 weeks with isotype control (Ø), Centotto-1 (C), anti-BAFF Sandy-2 (S), or both (C/S) (n= 5 mice per group, but one mouse was lost for FACS analysis in the control (Ø) group of panels C-F, and one mouse was lost in the anti-BAFF Sandy-2 (S) group of panels G-L). Note that mice immunized with cytochrome c and treated with

isotype control (Ø) is the control group shown on the right-hand side of graphs. **(A, G)** Titers of IgA, IgG and absorbance of IgM at a dilution of 1/2500 measured by ELISA in serum collected before treatment (day 0) or after 4 weeks of treatment (d28). **(B, H)** IgA, IgG, and IgM signals measured by ELISA at a single dilution (1:1, 1:25, 1:5, respectively) of bronchoalveolar fluids (BALF) or at a single dilution (1:20, 1:100, 1:100, respectively) of feces at day 28 of treatment. **(C, I)** Ratio of B/T cells measured by FACS in lymph nodes (LN). **(D, J)** Number of immature $B220^{int}$ B cells in the bone marrow (BM, one femur and one tibia). (**E, K**) Quantification of $CD138^{+}/B220^{+}$ plasmablasts and $CD138^{+}/B220^{-}$ plasma cells identified by FACS, normalized to immature B cells. **(F, L)** Quantification of IgA^{+} plasma cells in intestine (jejunum) and spleen sections stained by immunofluorescence for IgA and by DAPI for nuclei (ratio of $IgA^{+}/DAPI^{+}$). All panels: Line at median. One-way ANOVA with Dunnett's multiple comparison test. Ns: not significant. * P < 0.05. ** P<0.01. *** P<0.001. This experiment was performed once. Note that further analysis of mice in panels G-L is reported in Figure 3.

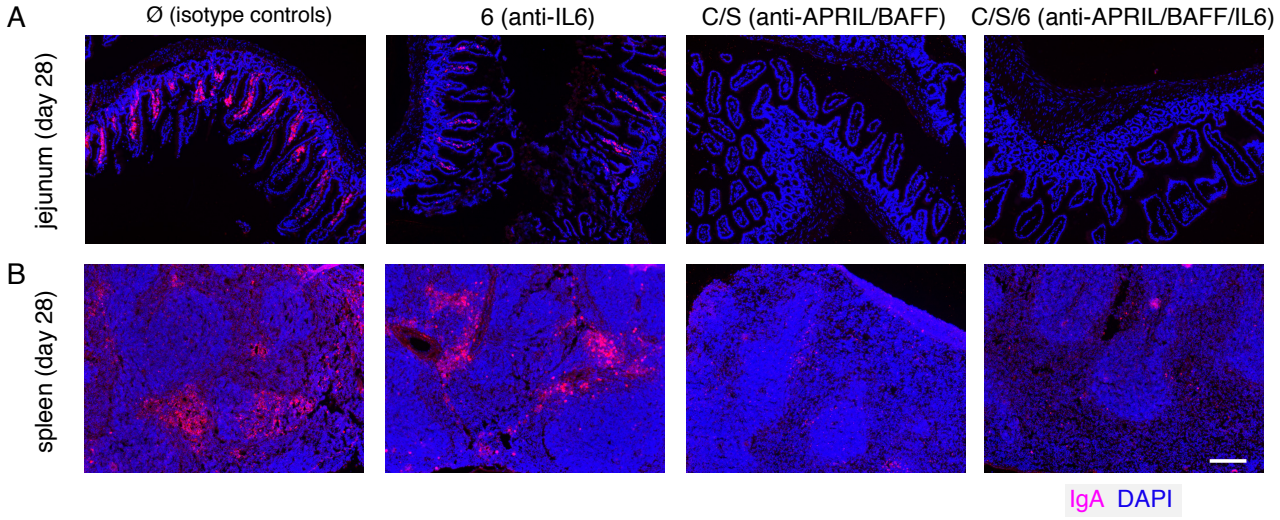


Ø = Isotype control. C= Centotto1 anti-APRIL. S = Sandy2 anti-BAFF.

Supplementary figure S4 | Related to figure 4. Inhibition of APRIL and BAFF in ligand-deficient mice. BAFF-ko and APRIL-ko mice were treated with isotype control (Ø), anti-APRIL Centotto-1 (C) or anti-BAFF Sandy-2 (S) for 4 weeks. Isotype control-treated WT and APRIL/BAFF double ko mice were also analyzed (n= 5 per group). APRIL-ko, BAFF-ko and WT + double ko were analyzed in independent cohorts and are not necessarily directly comparable. **(A)** IgA, IgG, and IgM signals measured by ELISA at a single dilution (1:1, 1:25, 1:5, respectively) of bronchoalveolar fluids (BALF) at 4 weeks. **(B)** Quantification of $CD138^{+}/B220^{+}/TACI^{+}$ plasmablasts identified by FACS, normalized to immature B cells ($B220^{int}$). **(C)** Quantification of IgA^{+} plasma cells in spleen sections stained by immunofluorescence (IF) for IgA and by DAPI for nuclei (ratio of $IgA^{+}/DAPI^{+}$). Lines indicate median. One-way ANOVA with Dunnett's multiple comparison test. Ns: not significant. * P < 0.05. ** P<0.01. *** P<0.001.



Supplementary figure S5 | Related to figure 5. Inhibition of APRIL and BAFF in receptor-deficient mice. BCMA-ko, TACI-ko and BCMA/TACI double ko mice were treated with isotype control (Ø), anti-APRIL Centotto-1 (C) anti-BAFF Sandy-2 (S) or both (C/S) for 4 weeks (n= 5 per group, except double ko n = 4 per group). BCMA-ko, APRIL-ko and double ko mice were analyzed in independent cohorts and are not necessarily directly comparable, but all groups contained a control group of wild type mice (n = 5) treated with the isotype control. **(A)** IgA, IgG, and IgM titers measured by ELISA in serum before treatment initiation. **(B)** IgA, IgG, and IgM signals measured by ELISA at a single dilution (1:1, 1:25, 1:5, respectively) of bronchoalveolar fluids (BALF) at 4 weeks. **(C)** Quantification of $CD138^+/B220^+$ (or $CD138^+/B220^+/TACI^+$ for BCMA-ko) plasmablasts identified by FACS, normalized to immature B cells ($B220^{int}$). Lines indicate median. One-way ANOVA with Dunnett's multiple comparison test. Ns: not significant. * P < 0.05. ** P<0.01. *** P<0.001.



Supplementary figure S6 | Related to figure 6. Representative immunofluorescence staining of IgA (red) and DAPI for nuclei (blue) in **(A)** jejunum and **(B)** spleen sections of FcRn (*fcgrt*)-deficient mice treated with isotype controls (Ø), anti-IL-6 (6), anti-APRIL/anti-BAFF Centotto-1/Sandy-2 (C/S) or all three inhibitory antibodies (C/S/6) for 4 weeks. Scale bar: 50 µm.

Supplementary Tables

Table S1. Plasmids used in this study

Plasmid	Designation	Protein encoded	Vector
ps515	EGFP	Enhanced green fluorescent protein	PCR3-Zeo
ps2309	hBCMA : Fas	Ig signal-VQCEVKLVPRGS-hBCMA (aa 2–54)-VD-hFas (aa 169–335)	ps1377 pMSCV
ps3618	Flag mAAA	HA signal-Flag-GPGQVQLQ-mAPRIL (aa95-232)-GGGGS- mAPRIL (aa95-232)-GGGGS-mAPRIL (aa 92-232)	PCR3
ps3637	Flag-mBAA	HA signal-Flag-GPGQVQLQVD-mBAFF (aa 81-309)-GGGGS-mAPRIL (aa 92-232)-GGGGS-mAPRIL (aa 92-232)	PCR3
ps3638	Flag-mBBB	HA signal-Flag-GPGQVQLQVD-mBAFF (aa 81-309)-GGGGS-mBAFF (aa 81-309)-GGGGS-mBAFF (aa 81-309)	PCR3
ps3640	Flag-mBBA	HA signal-Flag-GPGQVQLQVD-mBAFF (aa 81-309)-GGGGS-mBAFF (aa 81-309)-GGGGS-mAPRIL (aa 92-232)	PCR3

Ig signal = MNFGFSLIFLVLVLKG, HA signal = MAIIYLILLFTAVRG
Flag = DYKDDDDK, aa = amino acids

Table S2. Antibodies and reagents used for flow cytometry

#	Antigen recognized	Conjugate	Provider / catalogue number/ clone / working dilution
1	B220	FITC	eBioscience / #11-0452-82 / clone RA3-6B2 / 1:300
2	B220	AF700	Biolegend / #103232 / clone RA3-6B2 / 1:300
3	B220	APC-Cy7	Biolegend / #103224 / clone RA3-6B2 / 1:300
4	CD3	APC	eBioscience / #17-0032-82 / clone 17A2 / 1:100
5	CD3ε	PECy5.5	eBioscience / #35-0031-82 / clone 145-2C11 / 1:250
6	CD138	PECy7	Biolegend / #142513 / clone 281-2 / 1:100
7	CD138	BB515	BD Bioscience / #564511 / clone 281-2 / 1:300
8	CD19	APC	Invitrogen / # RM7705 / clone 6D5 / 1:200
9	CD19	PECy7	eBioscience / #25-0193-82 / clone eBio1D3 / 1:200
10	CD19	AF700	Biolegend / #115528 / clone 6D5 / 1:250
11	TACI	PE	R&D systems / #FAB1041P / clone 166010 / 1:300
12	IgA	PE	SouthernBiotec / #1165-09 / 1:300
13	IgG	APC	SouthernBiotec / #1030-31 / 1:600
14	IgM	BV421	BD Bioscience / #743323 / clone II/41 / 1:300
15	Live dead	Zombie aqua	Biolegend / #423102

Table S3. FACS staining mixes

Purpose	Antibody mix (See Table S2)	Used for mouse cohorts[#]
B & PC in BM	1 / 6 / 8 / 11	WT w9 / APRIL / BAFF
B & PC in BM	2 / 7 / 9 / 11	BCMA / TACI / BCMA-TACI / APRIL-BAFF
B & T in LN	1 / 4	WT w9 / APRIL / BAFF / APRIL-BAFF / TACI
B & T in LN	2 / 4	BCMA / BCMA-TACI
B, T, & PC A/G/M in LN, spleen, BM	3 / 5 / 7 / 10 / 12-15	FcRn

[#] All mice cohorts contained untreated or isotype control-treated WT mice. B: B cells. PC: plasma cells. T: T cells. BM: bone marrow. LN: lymph nodes. A/G/M: IgA, IgG, and IgM isotypes. w9: week 9.

Supplementary Methods

Study design

5 mice per group was used throughout the study, except for one experiment with 4 mice per group due to mice availability. Sample size of 5 animals per group was determined prior to the study using G*Power 3.1 to detect, with a power of greater than 0.8, differences of 50% between means when variation coefficient (= standard deviation/average*100) is 20%, α is 0.05, number of groups per experiment is 4 or 5, and data are normally distributed with equal variance. Rules for stopping data collection was determined in advance, but time of endpoint analysis was chosen to be 4 weeks post-treatment initiation according to results of the first time-course experiment. All data is included, but outliers are shown in parentheses and results of statistical analysis is also shown (in parentheses) without outliers. Each experiment was usually performed once, but main data were substantiated by repetitions under a range of conditions.

The research objective was to identify pairs of ligand – receptors in the BAFF – APRIL – BAFFR – TACI – BCMA system that can support plasma cells and therefore IgA, IgG and IgM levels in different compartments, by combining genetic and pharmacological approaches, the later achieved with high quality ligand antagonist unlikely to generate anti-drug antibody responses. Another objective was to compare contributions of IL-6 and APRIL/BAFF. Objectives added during the course of the study were i) to remove confounding contribution of neonatal Fc receptor and ii) to differentiate IgA, IgG and IgM plasma cells. Experiments were performed in mice, in controlled laboratory experiments.

Naïve or immunized mice were treated with ligand antagonists or isotype controls, alone or in combination, so that amounts of antibody administered were constant (except one experiment performed with buffer instead of isotype control). Antibody levels and the presence of plasma cells were determined usually after 4 weeks of treatment. Cages with mice of different genotypes, or males versus females were not mixed, but a mix of different treatments was assigned to each cage, and then randomly to mice in the cage (based on mouse ID). Males and females were distributed so that each treatment group contained both. The experiment was not blinded to investigators (except for experiments to quantify antibodies) and neither was analysis.

Genotyping

Ear biopsies were digested for 3 to 16 h at 55°C in 50 µl of DirectPCR Lysis Reagent (Peqlab, #31-102-T) with 2 µl of Proteinase K stock solution (Roche, #03115828001), heated for 45 min at 85°C, centrifuged and stored at 4°C. PCR primers were as follows. APRIL ko: wt 3'-CTCCAGACTGCCTTGGGAAAA-5', mutant 3'-TGGATCAGTAGTGCGACAGC-5' and shared 3'- TCTTCCCTTCTGTGCCTTTG -5'; BAFF ko: wt 3'-CAAGTTGATGTCCTGACCC AAGGCACC-5', mutant (neo cassette) 3'-TGGCAGGGTCTTTGCAGACTCATCCAT-5', shared 3'-GCAGATTGAGCAATCCATGGAAGGCCA-5'; TACI ko: wt 3'-TTTCCTTGAGG AGTCCCAGTACTGATC -5', mutant 3'-GATATCCTGATCATCGGTCTTCAGATGC-5' and shared 3'-AGGACAATGTGGGGACAGTCAGGGTAC-5'; BCMA ko: wt 3'-TCACTGTGGA AACACTGTTGCGCCATG-5', mutant (neo cassette) 3'-GATATCCTGATCATCGGTCTTCA GATGC-5' and shared 3'- CTGTCCACATTGCAACTGTTACCTGGG -5'; Fcgrt (FcRn)-ko: wt 3'-GGGATGCCACTGCCCTG-5', mutant 3'-GGAATTCCCAGTGAAGGGC-5', shared 3'-CGAGCCTGAGATTGTCAAGTGTATT-5'. Reactions contained 1 µl genomic DNA sample (ear lysate), 10 µl of GoTaq Green MMix 2x (Promega, #M7122), 3 x 1 µl of primers at 10 µM and 6 µl water. Amplification programs were 30 cycles of 94°C (7 sec)/60°C (20 sec)/72°C (20 sec) for APRIL ko, BAFF ko and TACI ko, 30 cycles of 94°C (7 sec)/58°C (20 sec)/72°C (35 sec) for BCMA ko and 30 cycles of 94°C (7 sec)/63°C (20 sec)/72°C (20 sec) for Fcgrt (FcRn)-ko.

Transfection of HEK 293T cells

Confluent HEK293T cells in DMEM 10% fetal calf serum were diluted 8-fold in 8 ml medium in 10 cm diameter culture plates and let to attach for 6 - 8 h. 7 µl of plasmid of interest at 1 µg/ml, 1 µl (1 µg) of EGFP plasmid tracer in 1 ml of DMEM medium without serum and antibiotics were mixed with 21 µl of a polyethylenimide (PEI) solution at 1 mg/ml (*67*), incubated for 15 min and transferred into the pate of HEK293T cells. After an

overnight incubation at 37°C and 5% CO2, cells were washed with PBS and medium replaced with 8 ml of serum-free OptiMEM medium (Invitrogen, #51985-042). 6 days later, conditioned supernatant was harvested, concentrated 20-fold by ultrafiltration using 30 kDa cut-off concentrators and stored at -70°C until use.

ELISA

Titers of IgA, IgM and IgG were measured by sandwich ELISA. MaxiSorp ELISA plates were coated with goat anti-mouse IgM µ chain-specific (Jackson, #115-006-075), goat anti-mouse IgG γ chain-specific (Jackson, #115-005-071), goat anti-mouse IgA (Novus #NB7501) or NP-25 BSA (Biosearch Technologies, #N5050-100) at 1 µg/ml in 50 µl of PBS overnight at room temperature, blocked with PBS, 4% powdered skimmed milk, 0.5% Tween-20 for 1 h at 37°C, then washed twice with PBS 0.05% Tween-20. Sera at a dilution of 1/250 and BALF and feces at 1/2 in incubation buffer (PBS, 0.4% milk, 0.05% Tween20) were diluted in serial 5-fold dilutions in independent plates, then 50 µl were transferred to the coated ELISA plates and incubated for 1 h at 37°C. Where indicated, measures were performed at a single sample dilution (BALF 1/1, 1/25 and 1/5 for IgA, IgG, and IgM; feces at 1/20 for IgA and 1/100 for IgG and IgM). Further steps were: three washes; 50 µl of biotinylated anti-mouse IgA (Caltag, #M31015) or biotinylated anti-mouse IgM (Jackson, #115-065-075) or biotinylated anti-mouse IgG (H+L) (Jackson, #115-065-062), all at 1/5000 in incubation buffer for 1 h at 37°C; three washes; 50 µl of horseradish-coupled streptavidin (Jackson, #016-030-084) at 1/4000 for 45 min at 37°C; five washes; 100 µl of OPD solution (Sigma, #P9187-50SET). After adequate color development, reaction was stopped with 50 µl of 1M HCl. Absorbance was monitored at 492 nm with a Halo LED microplate reader.

Flow cytometry

The spleen was weighed and cut into two pieces. One half was weighed again and mashed in DMEM 10% fetal calf serum through a 40 µm cell strainer (Falcon, Cat #352340) using the plunger of a syringe. Cells were spun at 4°C for 5 min at 1500 rpm (400 x g), resuspended in 1 ml of red blood cell lysis buffer (10 mM $NaHCO_3$, 150 mM NH_4Cl, 100 µM Na_2-EDTA, pH 7.4) for exactly 5 min, diluted with 7 ml of PBS 5% fetal calf serum, spun at 4°C for 5 min at 1500 rpm (400 x g), resuspended in 500 µl of DMEM 10% fetal calf serum, filtered through a nylon mesh, counted with a VICELL XR cell counter (Beckman) and used for FACS analysis. Lymph nodes (inguinal, axillary, cervical) were similarly prepared, omitting the red blood cell lysis step. Bones from one leg (femur and tibia) were cut at both extremities and placed in a 0.5 ml tube with a hole at the bottom. This tube was inserted in an uncapped 2 ml tube containing 200 µl of DMEM 10% fetal calf serum, then spun for 1 min at 13300 rpm (17000 x g). Collected cells were then processed at described for the spleen. Cells (2 x 10^6) spun in round-bottom 96 well plates were incubated with 10 µl of anti-CD16/32 cl93 at 1:100 in FACS buffer (eBioscience, #14-0161-82) to block Fc receptors, then stained with 20 µl of a mix of anti-CD138-PECy7, anti-B220-FITC, anti-CD19-APC, anti-TACI-PE, or alternatively with a mix of anti-CD138-BB515, anti-B220-AF700, anti-CD19-PECy7, anti-TACI-PE to stain B cells and plasma cells in bone marrow samples. A mix of anti-B220-FITC (or alternatively anti-B220-AF700) and anti-CD3-APC was used to stain B and T cells in lymph node samples (Table S2, S3). For the FcRn-deficient cohort, a single mix of anti-CD138-FITC, anti-B220-APC-Cy7, anti-CD3-PECy5, anti-CD19-AF700, anti-IgA-PE, anti-IgG-APC, anti-IgM-BV421 and of live dead zombie aqua (the latter being a marker for live versus dead cells) was used for lymph node, bone marrow and splenic cells. Following that, cells were permeabilized using BD Cytofix/CytopermTM Fixation/Permeabilization kit (BD, #554714) according to manufacturer's instructions. Intracellular staining was performed with a single mix containing anti-IgA-PE, anti-IgG-AF647, anti-IgM-BV421. Antibodies and working concentrations are described in Table S2 and Table S3. Cells were washed, fixed for 20 min in Fix/Perm buffer (BD, #554714), then washed with PBS 5% fetal calf serum. 500'000 events were acquired using a Cytoflex S B3-R3-V4-Y4 (Beckman Coulter). Data was analyzed with FlowJo version 10.9.0 (Beckton Dickinson).

Immunofluorescence

The portion of the intestine between the stomach and cecum was freed from mesenteric tissues, flushed with PBS, separated into duodenum (proximal third), jejunum (mid portion) and ileum (distal third), placed on a humidified blotting paper, opened along the mesenteric line so that the lumen faces up. With a wooden barbecue stick, the intestine was rolled so that the luminal side faced outwards. Intestine rolls and half of the spleens were fixed overnight at 4°C in 1% paraformaldehyde, then washed twice in PBS and incubated overnight at 4°C in 30% sucrose. The stick was then removed prior to embedding intestine rolls and half spleens in OCT, freezing in a bath of dry ice in ethanol and storing at -70°C until use. Spleen and intestine rolls in OCT were cut into 6-8 µm thick sections. Slides were pre-wet in PBS with two incubations of 10 min, then blocked with PBS 1% BSA, 1% normal mouse serum, 1% normal donkey serum for 20 min at room temperature and stained with goat anti-mouse IgA (Novus, #NB7501) at 1:400 in PBS 1% normal mouse serum, 0.1% BSA overnight at 4°C. Slides were washed in PBS and stained with donkey anti-goat-Cy3 (Jackson, #705-165-147) at 1:400 in PBS 1% normal mouse serum, 0.1% BSA for 45 min at room temperature. Slides were washed and stained with 3.3 µg/ml of 4',6-diamidino-2-phenylindole (DAPI) in PBS 1% normal mouse serum, 0.1% BSA for 5 min at room temperature and washed twice with PBS. Slides were mounted and three pictures were taken from each organ using an Axiovision 2B microscope. The ratio of IgA+ (Cy3-stained) area to DAPI+ area was determined using Image J (Fiji). The threshold of one image in 8-bit format for each Cy3 and DAPI fluorescence was set using Image / Adjust / Threshold so that selected area matched visible staining. The same threshold was applied to every image. The percentage of total and fluorescent areas (Analyze / Set measurements) was then determined (Analyze / Measure) and used to calculate the IgA+ area to DAPI+ area ratio.

ELISPOT

ELISPOT filter plates (Millipore #MSIPS4510) were coated overnight at 4°C with 100 µl per well of (4-hydroxy-3-nitrophenyl) (NP)25-BSA (Biosearch, #N-5050-100) at 5 µg/ml in 50 mM Na carbonate pH 9.6, then washed twice with PBS. Plates were blocked with 300 µl of DMEM, 10% fetal calf serum for 2 h at 37°C. Bone marrow cells (800'000 cells in 100 µl medium) were added in triplicates and left overnight at 37°C, 5% CO_2. Plates were then washed 3 times with PBS 0.05% Tween-20 and twice with PBS, incubated for 2 h at room temperature with 100 µl of goat anti-mouse IgG (H+L) (Jackson ImmunoResearch #115-035-146) (1/5000 in PBS 1% BSA), washed 3x with PBS 0.05% Tween-20 and twice with PBS, incubated with 100 µl of AEC solution until adequate color development. Plates were washed with water, dried overnight at room temperature in the dark, and spots were counted manually using a binocular with 20-fold magnification. The AEC solution was prepared like this: 3-amino-9-ethylcarbazole (Sigma #A5754) was dissolved at 10 mg/ml in N,N-dimethylformamide, then further diluted to 0.33 mg/ml in 0.1 M Na-acetate pH 5, and filtered at 0.45 µm. 30% H_2O_2 was added at 1/2000 just before use to reach a final H_2O_2 concentration of 0.015%.