Impaired IgA class switching in APRIL-deficient mice

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Communicated by Frederick W. Alt, Harvard Medical School, Boston, MA, January 14, 2004 (received for review August 21, 2003)

The tumor necrosis factor (TNF) family member APRIL binds to the receptors BCMA on B cells and TACI on B and T cells. To investigate the role of APRIL in immunity, we generated APRIL-deficient mice. APRIL$^{-/-}$ mice have normal T and B lymphocyte development, normal T and B cell proliferation *in vitro*, but increased numbers of CD44^{hi}CD62L^{lo} CD4^{+} effector/memory T cells and increased IgG responses to T-dependent antigens. Serum IgA levels were significantly decreased, and serum IgA antibody responses to mucosal immunization with TD antigens and to type 1 T-independent antigens were impaired in APRIL$^{-/-}$ mice. APRIL by itself induced IgA as well as IgG1 isotype switching in CD40-deficient IgM^{+}IgD^{+} sorted B cells. These results suggest that APRIL down-regulates T cell-dependent antibody responses and promotes IgA class switching.

APRIL (TNFSF 13a), also known as TALL-2 and TRDL-1, and BAFF (TNFSF 13b), also known as BLyS, TALL-1, THANK, and zTNF4, are two recently described members of the tumor necrosis factor (TNF) family of ligands (1). They have a similar genomic organization and exhibit 50% homology at the protein level. APRIL and BAFF are synthesized as type II transmembrane proteins with a TNF homology C-terminal domain that is characteristic of TNF family members (1). Both APRIL and BAFF are proteolytically cleaved into a soluble form at a multibasic motif by a furin-like protease. APRIL is cleaved intracellularly, then secreted (2). Heterotrimers of APRIL and BAFF that can stimulate human B cells have been described (3). A TWEAK–APRIL fusion protein, TWE-PRIL, in which the APRIL extracellular domain is fused to the intracellular, transmembrane and part of the extracellular domains of TWEAK, is also known to exist (4).

APRIL is highly expressed in tumor cell lines and at different levels in a variety of tissues, including human monocytes/macrophages, dendritic cells, T cells, murine bone marrow, and spleen (4–7). APRIL and BAFF both bind to two receptors, B cell maturation antigen (BCMA) and transmembrane activator and calcium-modulator and cytophilin ligand interactor (TACI), which are members of the TNF receptor (TNFR) family (8–13). BCMA is exclusively expressed on B cells (14, 15), whereas TACI is expressed on B cells and activated T cells (16). A third receptor, BAFF-R (or BR3), which is unique for BAFF, is expressed on B cells (17, 18). An APRIL-specific receptor has been postulated based on the observation that some tumor cell lines bind APRIL but not BAFF (10, 19).

BAFF enhances B cell survival and synergizes with B cell receptor crosslinking to induce B cell proliferation *in vitro* (20–23). Mice treated with soluble BAFF and BAFF transgenic mice show increased number of mature B cells and plasma cells, enlarged secondary lymphoid organs, increased levels of polyclonal serum immunoglobulins of all isotypes, and increased number of germinal centers (GCs) in the absence of immunization (22, 24, 25). BAFF transgenic mice developed high titers of autoantibodies and a systemic lupus erythematosus-like condition (24). BAFF$^{-/-}$ mice have a severe defect in B cell development and virtually lack T_2 immature B cells, marginal zone B cells, and mature B cells (26, 27). These studies suggest that BAFF plays an important role in B cell activation and survival and is required for the transition from T_1 to T_2 immature B cells.

APRIL has been found to cause modest proliferation of B and T cells and to synergize with B and T cell receptor crosslinking to induce B and T cell proliferation *in vitro* (13, 19). Administration of murine soluble APRIL to mice results in increased spleen weight and increased percentages of splenic B cells (13). However, expression of a human APRIL transgene in mouse T cells resulted in no signs of B cell hyperplasia, but enhanced T cell survival *in vitro* and survival of staphylococcal enterotoxin B-reactive Vβ8^{+}CD4^{+} T cells *in vivo*; both correlated with elevated Bcl-2 levels (7). IgM, but not IgG, responses to TD antigens were increased in APRIL transgenic mice. The antibody response to type 2 T-independent (TI-2) antigen was enhanced (7). However, the role of APRIL in the development and function of immune cells remains to be defined. To this purpose we have generated and characterized APRIL-deficient mice.

Materials and Methods

Generation of APRIL$^{-/-}$ Mice. Murine APRIL cDNA was amplified by PCR from murine spleen cDNA (forward, primer 5′-CCTCACTTCTGAGACCACAGC-3′; reverse, primer 5′-GAACAACAGTCAAGGCAAAGC-3′), and used to isolate genomic DNA fragments encoding the murine APRIL gene from a Lambda FIXII library obtained from the 129Sv mouse strain (Stratagene). The targeting construct was assembled in the pLNTK targeting vector (28) with the NEO cassette in the opposite orientation of the APRIL gene and replacing half of exon 3 and all exons 4–6 that encode for the last 126 aa of the 201-aa-long extracellular domain of mAPRIL. This substitution modified the *Bam*HI restriction map of this area allowing the detection of the targeted gene via Southern blot analysis. A copy of the thymidine-kinase gene for negative selection was present at the 5′ end of the construct (Fig. 1*A*). The linearized construct was transfected into J1 embryonic stem (ES) cells, which were selected in medium containing 0.4 mg/ml G418 and 10 μg/ml gancyclovir. Five clones were identified to contain a normal and a disrupted allele and no random integration of the *neo* gene (Fig. 1*B*). ES clones were injected into 3.5-day-old C57BL/6 blastocysts and APRIL$^{-/-}$ mice were obtained by standard methods (29).

RT-PCR Analysis. Total RNA extracted from spleen and bone marrow by using TRIzol (Invitrogen) was reverse-transcribed by SuperScript II (Invitrogen) according to manufacturer's instructions. The primers for APRIL were as described above. For α, ε, and γ1 germ-line (GLT) and mature (pST) transcripts, activation-induced deaminase (AID), and β_2-microglobulin, the primers used were as described (30, 31). For other genes, the primers were as follows: TWEAK forward, 5′-GTGCTGAGCCTGGGCCTG-3′; TWEAK reverse, 5′-CTGGGAGAGCAAGGCCC-3′; Smt3ip-1 forward, 5′-GATGAAAGAGACTATACAGG-3′; Smt3ip-1 reverse, 5′-CTCTACAACTTCATCAGTGC-3′. Amplified products were analyzed on agarose gel. Various dilutions of cDNA were used to ensure that the products measured were in the linear range.

Abbreviations: TNF, tumor necrosis factor; BCMA, B cell maturation antigen; TACI, transmembrane activator and calcium modulator and cytophilin ligand interactor; GC, germinal center; TI-1, type 1 T-independent; TI-2, type 2 T-independent; ES, embryonic stem; GLT, germ-line transcripts; AID, activation-induced deaminase; LPS, lipopolysaccharide; TGF, transforming growth factor.

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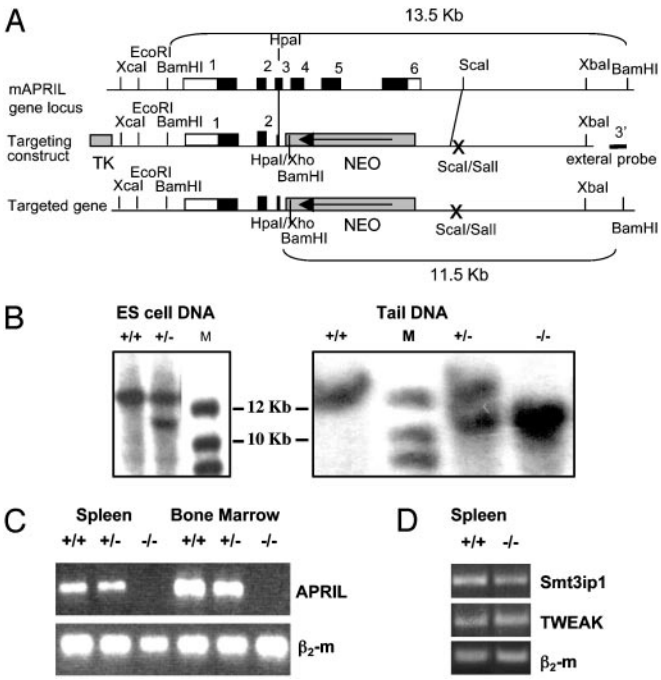

Fig. 1. Targeting the APRIL locus. (*A*) Partial restriction map of the *APRIL* gene (*Top*), *APRIL* targeting construct (*Middle*), and mutated *APRIL* allele after homologous recombination (*Bottom*). Exons are represented as black boxes. NEO, neomycin resistance gene; TK, thymidine kinase gene. A 0.65-kb fragment at the 3′ end of the gene was used as external probe. (*B*) Southern blot analysis of genomic DNA from untransfected (+/+) ES cells and heterozygous (+/−) clone (*Left*) and from tails of WT (+/+), heterozygous (+/−), and homozygous (−/−) APRIL mutant mice (*Right*). Ten micrograms of genomic DNA were digested with *Bam*HI and hybridized with the 3′ external probe. The upper band (13.5 kb) indicates the WT allele, and the lower band (11.5 kb) corresponds to the targeted allele. (*C*) RT-PCR analysis of APRIL mRNA expression in spleen and bone marrow of WT (+/+), heterozygous, (+/−), and homozygous (−/−) APRIL mutant mice. RT-PCR of β_2-microglobulin (β_2m) was used as control. (*D*) RT-PCR of TWEAK and Smt3ip-1 mRNA expression in spleens of WT (+/+) and APRIL mutant (−/−) mice.

Antibodies and Flow Cytometry Analysis. mAbs to the following mouse antigens were purchased from BD Pharmingen: CD3ε, CD4, CD8α, CD44, CD62L, B220, CD43, CD40, IgM, IgD, CD23, CD27, CD21, CD25, CD69, and CD5. Single-cell suspensions from different tissues were stained with FITC-, phycoerythrin-, or Cy-Chrome-conjugated antibodies in PBS containing 5% rat serum (Sigma), 0.01% sodium azide, and Fc-block (Pharmingen), and analyzed on a FACSCalibur flow cytometer (BD Pharmingen) as described (28).

Proliferation and Ig Secretion by B Cells. B cells were purified by negative selection of spleen cells using biotinylated anti-(α)-CD43 mAb and streptavidin coated M-280 Dynabeads. B cells (>85% B220$^+$) were cultured in RPMI medium 1640 containing 10% FCS, L-glutamine, and 50 μM 2-mercaptoethanol (medium) at 0.5×10^6 per ml with α-IgM (10 μg/ml), α-CD40 (1 μg/ml), lipopolysaccharide (LPS 10 μg/ml), with or without IL-4 (50 ng/ml, R & D Systems). After 72 h, cultures were pulsed with 1 μCi of [^{3}H]thymidine for 16 h, harvested, and scintillation counted (1 Ci = 37 GBq). For Ig synthesis, B cells were cultured at 1×10^6 per ml with LPS+IL-4 and α-CD40+IL-4 for 6 days, and IgG1 and IgE were assayed in the supernatants by ELISA.

Isotype Switching in B Cells. Spleen cells from CD40$^{-/-}$ mice were sorted by FACSVantage SI for IgM$^+$IgD$^+$ B cells with a purity of >93%. Cells were cultured in medium at 1×10^6 per ml alone, or in the presence of 1 μg/ml soluble APRIL (Alexis or R & D Systems), 1 μg/ml soluble BAFF (Alexis), 10 μg/ml LPS with 50 ng/ml IL-4 or 1 ng/ml transforming growth factor (TGF) β (R & D Systems). Day 6 supernatants were collected and assayed for IgA, IgE, IgG1, IgG2a, IgG2b, and IgG3 by ELISA. Genomic DNA was prepared from cells with standard procedure. For RNA preparation, cells were cultured for 4 days, and total RNA and cDNA were prepared as described above.

ELISAs. Serum Ig levels were assayed by ELISA as described (28). To measure α-NP or α-chicken gamma globulin (CGG)-specific antibodies, plates were coated with 50 μg/ml NP_4- or NP_{25}-BSA or CGG (Biosearch Technologies). Alkaline phosphatase-conjugated isotype-specific antibodies (Southern Biotechnology Associates) were used as revealing antibodies.

Immunization, Antibody Titers, and Measurement of Affinity Maturation. Twelve- to 16-week-old mice, five APRIL$^{-/-}$ and five WT, were immunized i.p. with 200 μg of an alum precipitate of NP_{28}-CGG (Biosearch Technologies) containing pertussis toxin (300 ng per mouse, List Biologicals). Mice were boosted i.p. 14 days later by using 30 μg of NP_{28}-CGG in aqueous solution. Mice were killed at day 21, sera were collected, and spleens were frozen in OCT compound (Ames). A second group of mice, seven APRIL$^{-/-}$ and seven WT, received 100 μg of NP-LPS (Biosearch Technologies), and a third group, six APRIL$^{-/-}$ and five WT mice, received 250 μg of NP-FICOLL (Biosearch Technologies). Immunization protocols were as described (28). Sera were collected at day 14, and serial dilutions were analyzed for NP isotype-specific antibodies by ELISA. A fourth group of mice, five APRIL$^{-/-}$ and five WT, were i.p. primed with 100 μg of CGG, followed by four boosts with 10-day intervals of 50 μg of CGG plus 1 μg of Cholera toxin subunit B (CT) by intranasal (i.n.), and 250 μg of CGG plus 5 μg of CT by intragastric (i.g.) injections. At day 48, sera were collected and analyzed for CGG isotype-specific antibodies by ELISA.

To determine NP-specific high-affinity and total (high- and low-affinity) antibodies, plates were coated with NP_4-BSA (for high-affinity) and NP_{25}-BSA (for high- and low-affinity). An increase in the ratio NP_4/NP_{25} demonstrates affinity maturation (32).

Immunohistochemistry. GCs were detected in 4-μm-thick frozen spleen sections, stained with biotin-labeled peanut agglutinin (Vector Laboratories) followed by avidin-biotinylated peroxidase complex (Dako), and developed with 3-amino-9-ethylcarbazole (Sigma). IgA$^+$ plasma cells were detected in frozen sections of the small intestine stained with rat α-mouse IgA (BD Pharmingen) followed by biotinylated rabbit α-rat IgG and developed by the immunoperoxidase method.

Digestion Circularization (DC)-PCR. Genomic DNA isolated from cultured splenic B cells on day 6 was digested with *Eco*RI, circularized, and used as template for PCR using primers as reported for Sμ-Sγ1, Sμ-Sε, and nicotinic acetylcholine receptor β unit (nAChR) (30) and for Sμ-Sα (33). Five nanograms of circular DNA was amplified for Sμ-Sγ1 and nAChR, and 20 ng was amplified for Sμ-Sα and Sμ-Se.

Statistical Analysis. Statistical analysis was conducted by using PRISM software (GraphPad, San Diego).

Results

Generation of APRIL Mutant Mice. The partial restriction map of *APRIL* gene, targeting construct, and targeted allele is shown in Fig. 1*A*. ES clones with targeted disruption of one *APRIL* allele were identified by the presence of an 11.5-kb *Bam*HI fragment derived from the targeted allele and a 13.5-kb *Bam*HI fragment derived from the WT allele (Fig. 1*B Left*) and were used to generate APRIL$^{-/-}$ mice, which were identified by Southern blot analysis (Fig. 1*B Right*). Analysis of cDNA from spleen and bone marrow

cells showed that APRIL$^{-/-}$ mice have no detectable full-length coding APRIL mRNA (Fig. 1*C*). Expression of TWEAK and Smt3ip-1 genes, which flank within ≈1 kb, was normal in APRIL$^{-/-}$ mice (Fig. 1*D*). APRIL$^{-/-}$ mice were born at the expected Mendelian ratio and did not display differences from APRIL$^{+/+}$ (WT) littermates in growth, weight, or health (data not shown).

APRIL$^{-/-}$ Mice Have Normal Lymphoid Development. No significant differences were found in the size or cellularity of thymus, spleen, axillary and mesenteric lymph nodes between APRIL$^{-/-}$ mice and WT littermates analyzed at 10–16 weeks of age (data not shown). Bone marrow from APRIL$^{-/-}$ mice had normal percentages of B220^{+}cells with a normal expression profile of IgM, and CD43, and the percentages of B220^{+}, CD5^{+} B1 cells in the peritoneum of APRIL$^{-/-}$ mice were comparable to those in WT controls (data not shown). FACS analysis of thymocytes at 12 weeks of age revealed no differences in the percentages of CD4^{-}CD8^{-}, CD4^{+}CD8^{+}, CD4^{+}, and CD8^{+} cells. Spleens from APRIL$^{-/-}$ mice had normal percentages of CD3^{+}, CD4^{+}, and CD8^{+} cells and of B220^{+}, IgM^{+} IgD^{+} CD40^{+}, CD21^{+}, CD23^{+}, and CD27^{+} cells (data not shown). However, CD4^{+}, but not CD8^{+} splenic T cells from APRIL$^{-/-}$ mice had significantly increased percentages of CD44^{hi}CD62L^{lo} effector/memory T cells (28.4 ± 3.2 in APRIL$^{-/-}$ mice versus 17.5 ± 2.0 for WT mice; $P < 0.02$ by Student *t* test, $n = 5$ for each group). Peripheral and mesenteric lymph nodes and peripheral blood lymphocytes also showed normal percentage of B220^{+}, CD3^{+}, CD4^{+}, and CD8^{+} cells (data not shown). These results indicate that APRIL is not required for T and B lymphocyte development, but suggest that it may regulate effector/memory T cell numbers.

Normal T and B Cell Proliferation and *in Vitro* Ig Production in APRIL$^{-/-}$ Mice. Proliferation and expression of the activation markers CD25 and CD69 by splenocytes and purified T cells (>85% CD3^{+}) from APRIL$^{-/-}$ mice in response to plate-bound α-CD3 was comparable to WT controls (data not shown). Purified B cells (>85% B220^{+}) from APRIL$^{-/-}$ mice showed normal proliferation in response to α-IgM, LPS, and α-CD40 both in the presence or absence of IL-4 and secreted normal amounts of IgE and IgG1 in response to stimulation with α-CD40+IL-4 and LPS+IL-4 (data not shown).

Enhanced Antibody Responses to TD Antigens in APRIL$^{-/-}$ Mice. Twelve- to 16-week-old mice were immunized with the TD antigen NP_{28}-CGG, and the antibody response to the NP hapten was measured. APRIL$^{-/-}$ mice had normal IgM antibody responses, but significantly increased IgG antibody responses to NP in all four IgG subclasses (Fig. 2*A*). There was no difference in the IgA antibody response between APRIL$^{-/-}$ and WT mice. The affinity maturation of the IgG1 α-NP response in APRIL$^{-/-}$ mice was comparable to that of WT controls (Fig. 2*B*).

Spleens from unimmunized APRIL$^{-/-}$ mice and WT controls contained very few germinal centers (data not shown). After immunization with NP_{28}-CGG, spleens of APRIL$^{-/-}$ mice contained significantly more and larger GCs than WT controls (50.7 ± 14.0 in APRIL$^{-/-}$ mice, $n = 3$, compared to 15.4 ± 6.7 in WT controls, $n = 4$; $P < 0.005$ by Student's *t* test) (Fig. 2*C*). Taken together, these results suggest that APRIL normally regulates the formation of IgG antibody and GCs in response to TD antigens.

Decreased Serum IgA and Impaired IgA Antibody Responses to Mucosal Immunization in APRIL$^{-/-}$ Mice. Serum immunoglobulins concentrations were not significantly different between APRIL$^{-/-}$ mice and WT controls, except for IgA. Serum IgA was significantly decreased in APRIL$^{-/-}$ mice (mean ± SD: 48.4 ± 23.3 μg/ml vs. 102.4 ± 15.3 μg/ml in WT littermates; $P = 0.0003$ by Mann–Whitney test, Fig. 3). This suggests that APRIL plays a role in IgA production *in vivo*.

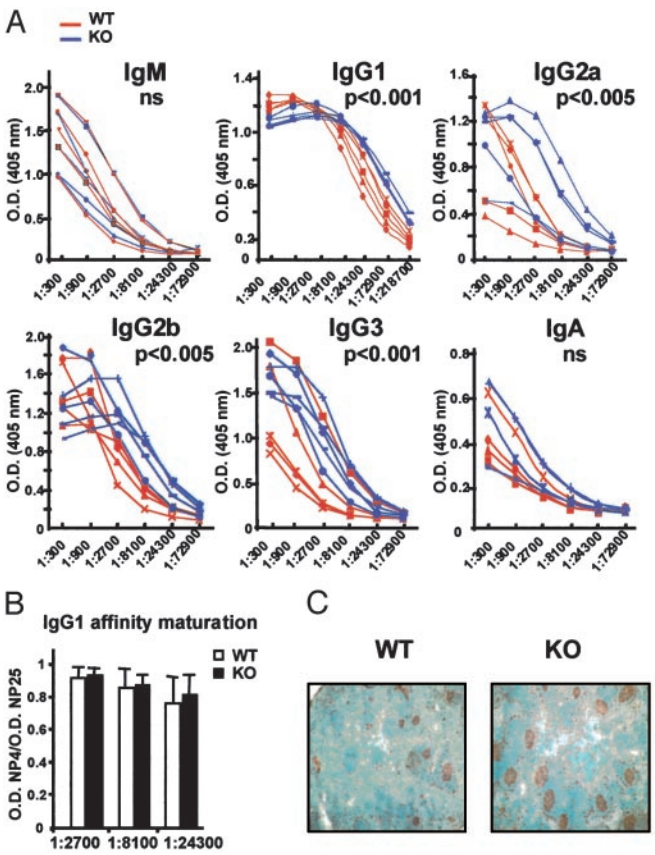

Fig. 2. Antibody response, affinity maturation, and germinal center formation in response to TD antigen in APRIL$^{-/-}$ mice. (*A*) IgM, IgG subclasses, and IgA antibody responses to NP after immunization with the TD antigen NP_{28}-CGG in APRIL$^{-/-}$ mice (KO, $n = 5$, blue curve; $n = 4$ for IgA) and controls (WT, $n = 5$, red curve; $n = 4$ for IgA). Statistical analysis was performed by using two-way ANOVA; ns, not significant. (*B*) Affinity of NP-specific IgG1 antibodies measured as ratio of the OD at 405 nm of the same serum dilution tested on NP_4-BSA- and NP_{25}-BSA-coated plates. The dilutions used were in the linear part of the titration curve. (*C*) Germinal centers in representative spleen sections (×40) from mice immunized with NP_{28}-CGG examined for PNA binding by immunohistochemistry.

Because a substantial fraction of circulating IgA is made in response to antigens encountered via the mucosal route, we examined the IgA antibody response of APRIL$^{-/-}$ mice to mucosal immunization. CGG antigen was administered to 12- to 16-week-old mice via the combined i.g. and i.n. routes with Cholera toxin B subunit as adjuvant. Fig. 4*A* shows that APRIL$^{-/-}$ mice made an IgM response to CGG equivalent to that of WT controls, but an IgG response that was significantly higher. In contrast, their IgA antibody response was significantly lower than that of WT controls. Examination of the small intestine revealed that the number of IgA^{+} plasma cells in the lamina propria of APRIL$^{-/-}$ mice was markedly reduced (Fig. 4*B*). There were 22.8 ± 3.0 IgA^{+} plasma cells per ×20 Power Field in the lamina propria of immunized APRIL$^{-/-}$ mice compared to 60.0 ± 6.8 in immunized WT controls ($n = 4$ for each group; $P = 0.001$ by Student's *t* test). Examination of mesenteric lymph nodes revealed no difference in the staining of CD3 or B220 between APRIL$^{-/-}$ mice and WT controls. However, there was decreased IgA staining in mucosally immunized APRIL$^{-/-}$ mice compared to WT controls (data not shown). Together, these data suggest that APRIL promotes IgA responses to antigens encountered via the mucosal route.

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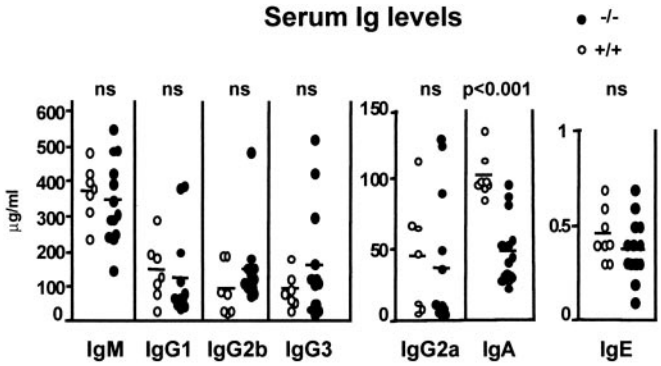

Fig. 3. Serum Ig in APRIL$^{-/-}$ mice. Serum Ig levels from 10- to 12-week-old nonimmunized APRIL$^{-/-}$ mice (n = 15 for IgA and IgE; n = 13 for the other isotypes) and WT littermates (n = 8 for IgA and IgE; n = 7 for the other isotypes). Bars represent the mean. Mann–Whitney test was used for statistical analysis (ns, not significant).

Impaired IgA Antibody Responses to Type I T-Independent (TI-1) Antigen. LPS-coated bacteria are major natural immunogens via the mucosal route. APRIL$^{-/-}$ mice made normal level of IgG3 α-NP antibodies in response to immunization with the TI-1 antigen NP-LPS. In contrast, their IgM and IgA α-NP response were significantly diminished (Fig. 4*C*). APRIL$^{-/-}$ mice made normal IgM, IgG3, and IgA α-NP responses to the TI-2 antigen NP-Ficoll (Fig. 4*D*). These results suggest that APRIL is required for the IgA antibody response to TI-1 antigens.

APRIL Induces IgA Class Switching in Murine B Cells. The deficient IgA antidody responses in APRIL$^{-/-}$ mice prompted us to examine its effect on IgA class switching. Because the APRIL homologue BAFF shares two of its three known receptors, namely TACI and BCMA, with APRIL, we examined in parallel the effect of BAFF on IgA switching. B cells used in these experiments were derived from CD40$^{-/-}$ mice and sorted for IgM and IgD expression to ensure that *de novo* switching rather than amplification of Ig synthesis by already switched B cells is being measured. APRIL by itself induced IgA, as well as IgG1 production by sorted CD40$^{-/-}$IgM^{+}IgD^{+} B cells, as well as IgG3 (Fig. 5*A* and data not shown). In contrast, it failed to induce IgE, IgG2a, or IgG2b production, but synergized with IL-4 to induce IgE synthesis (Fig. 5*A* and data not shown). Similar findings were obtained with BAFF. As expected, B cells synthesized IgA in response to LPS plus TGFβ, and IgG1 and IgE in response to LPS plus IL-4 (Fig. 5*A*). Similar results were obtained with B cells from WT mice sorted for IgM and IgD expression (data not shown). These results suggest that APRIL and BAFF induce, by themselves, IgA and IgG1 switching in naïve B cells.

The molecular events involved in isotype switching in naïve B cells include expression of germ-line transcripts, expression of the gene for AID, followed by deletional switch recombination and expression of mature Iμ-containing post switch transcripts (34). We examined the effect of APRIL on molecular events involved in IgA, IgG1 and IgE switching. APRIL by itself induced α and γ1GLTs, but not εGLT, and induced AID gene expression in CD40$^{-/-}$IgM^{+}IgD^{+} B cells (Fig. 5*B*). More importantly APRIL induced S$\mu \rightarrow$ Sα and S$\mu \rightarrow$ Sγ1, but not S$\mu \rightarrow$ Sε, deletional switch recombination (Fig. 5*C*), and mature α and γ1, but not εpST in these cells (Fig. 5*B*). Similar results were obtained with BAFF, although its ability to induce the molecular events involved in IgA and IgG1 switching was weaker than that of APRIL, consistent with its weaker ability to induce IgA and IgG1 secretion. As expected, TGFβ synergized with LPS to induce αGLT, S$\mu \rightarrow$ Sα deletional switch recombination, and mature α transcripts, whereas IL-4 synergized with LPS to induce γ1 and εGLT, S$\mu \rightarrow$ Sγ1 and S$\mu \rightarrow$ Sε deletional switch recombination, and mature γ1 and ε transcripts.

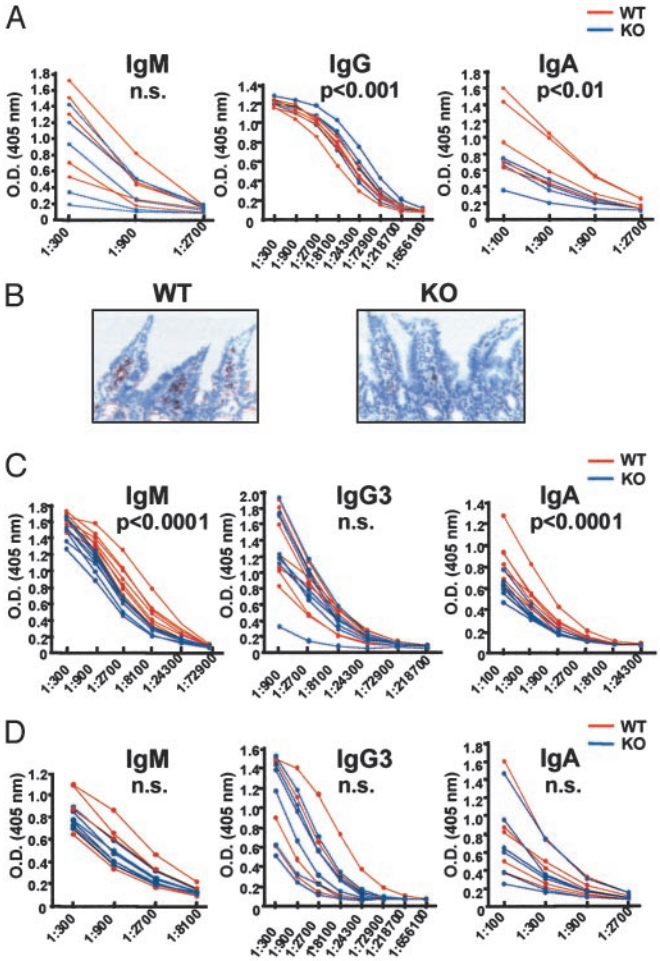

Fig. 4. IgA antibody responses to mucosal immunization and to TI antigen in APRIL$^{-/-}$ mice. (*A*) IgM, IgG, and IgA antibody responses to CGG of APRIL$^{-/-}$ mice, KO (n = 5) and WT littermates (n = 5) after mucosal (i.g. plus i.n.) immunization. Statistical analysis was performed by using two-way ANOVA. (*B*) Frozen sections of the small intestine (×20) from APRIL$^{-/-}$ (KO, *Right*) and WT mice (*Left*) stained for IgA by the immunoperoxidase method. The frozen sections shown are representative of four APRIL$^{-/-}$ or four WT control mice analyzed. (*C* and *D*) IgM, IgG, and IgA antibody responses to NP after i.p. immunization with the TI-1 (NP-LPS, seven WT mice and seven APRIL$^{-/-}$ mice) (*C*) or TI-2 (NP-Ficoll, five WT mice and six APRIL$^{-/-}$ mice) antigens (*D*). Statistical analysis was performed by using two-way ANOVA.

Discussion

The present findings suggest that APRIL plays an important role in IgA antibody responses to mucosally encountered antigens and to TI-1 antigens.

It was noted in recent reviews that disruption of the APRIL gene by one group has resulted in embryonic lethality, whereas its disruption by another group gave rise to viable mice (1, 35). The *APRIL* gene is closely flanked on the 5′ side by the gene encoding the TNF family member TWEAK and on the 3′ side by the gene encoding Smt3ip1/senp-3, a member of the sentrin/SUMO-specific protease family. Both genes are ≈1 kb from *APRIL*. It is possible that an APRIL-targeting construct may also affect the expression of these two genes, resulting in early embryonic lethality. Our APRIL-targeting construct disrupts the exons encoding for all of the β sheets of the EC domain of APRIL and resulted in viable APRIL$^{-/-}$ mice that completely fail to express full-length APRIL

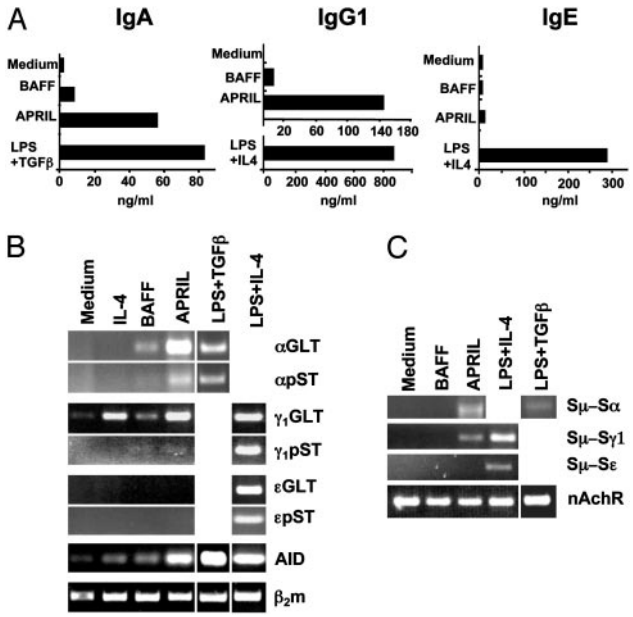

Fig. 5. APRIL and IgA class switching in murine B cells. (A) *In vitro* secretion of IgA, IgG1, and IgE by IgM^+IgD^+ B cells derived from CD40-deficient mice. Cells were stimulated for 6 days with APRIL, BAFF, LPS+TGFβ as control for IgA synthesis, and LPS+IL-4 as control for IgG1 and IgE synthesis. Similar results were obtained in two other experiments. (*B*) Expression of α, γ1 and εGLT, Iμ-Cα Iμ-Cγ1 and Iμ-Cε mature pST and AID transcripts measured by RT-PCR in B cells stimulated for 4 days with IL-4, APRIL, BAFF, and LPS plus TGFβ or LPS plus IL-4. RT-PCR for β2-microglobulin (β2-m) was used as control. (*C*) S$\mu \rightarrow$ Sα, S$\mu \rightarrow$ Sγ1, and S$\mu \rightarrow$ Sε deletional switch recombination measured by DC-PCR at day 6. DC-PCR for the acetylcholine receptor (nAchR) was used as control.

mRNA, but that express normal levels of TWEAK and Smt3ip1/senp-3 mRNA in their spleens. Our APRIL gene disruption strategy does not allow the formation of APRIL/BAFF heteromers nor the expression of TWE-PRIL protein, the lack of which may possibly contribute to the phenotype of our $APRIL^{-/-}$ mice.

$APRIL^{-/-}$ mice had a normal T cell phenotype in the thymus and periphery and normal T cell proliferation and IL-2R expression in response to α-CD3 stimulation. However, there was a consistent and significant expansion of $CD44^{hi}CD62L^{lo}$ effector/memory T cells in the spleen and lymph nodes. This suggests that APRIL regulates the expansion and/or survival of effector/memory T cells, possibly because it may deliver an apoptotic signal to activated T cells. In contrast to their expansion in $APRIL^{-/-}$ mice, $CD44^{hi}CD62L^{lo}$ T cells are reduced in $BAFF^{-/-}$ mice (27). This suggests that APRIL and BAFF exert opposing roles on the homeostasis of effector/memory T cells, possibly by means of the engagement of distinct receptors, such as BAFF-R and an APRIL-specific receptor that may be expressed on activated T cells (10, 16, 18).

The number and phenotype of B cells in the bone marrow, spleen, and peritoneum were normal in $APRIL^{-/-}$ mice. The finding that B cell development is normal in $APRIL^{-/-}$ mice is consistent with the observation that neither TACI nor BCMA are important for the development of mature B cells (36–38). However, TACI has been shown to play an important role in B cell homeostasis, because $TACI^{-/-}$ mice have increased numbers of B cells and develop a B cell lymphoproliferative disorder (38, 39). Because the B cell compartment was normal in size and distribution in $APRIL^{-/-}$ mice, BAFF and APRIL may have redundant roles in signaling by means of TACI.

$APRIL^{-/-}$ B cells proliferated normally *in vitro* in response to B cell receptor crosslinking, CD40 ligation, and LPS, and synthesized normal amounts of IgG1 and IgE in response to α-CD40+IL-4. However, $APRIL^{-/-}$ mice exhibit significantly increased serum IgG antibody responses and have increased numbers of germinal centers in their spleens after immunization with TD antigen. In contrast, their IgG3 antibody responses to TI-1 and TI-2 antigens were normal. These findings suggest that T cell help is enhanced in $APRIL^{-/-}$ mice. This may be related to the expansion of effector/memory cells. Further investigations are needed to examine T cell function in $APRIL^{-/-}$ mice. Because TACI is indispensable for TI-2 antibody responses (37, 38), the normal response of $APRIL^{-/-}$ mice to the TI-2 antigen NP-Ficoll suggests that APRIL and BAFF are redundant in delivering signals that are essential for antibody responses to TI-2 antigens via TACI.

$APRIL^{-/-}$ mice had a selective deficiency in serum IgA. This is unlikely to be due to disparity in genetic background between $APRIL^{-/-}$ and WT littermates because serum IgA levels were comparable in the two parental strains, C57BL/6 and 129/Sv (data not shown). Serum IgA is the only isotype that is only moderately, and not significantly, reduced in $BAFF^{-/-}$ mice, despite their low numbers of mature B cells (27). We have also observed no significant decrease in serum IgA in a line of $BAFF^{-/-}$ mice that we have independently generated (E.C., unpublished observation). In contrast, serum IgA is significantly reduced in $TACI^{-/-}$ mice (37). These findings, together with the fact that serum IgA levels are not diminished in $CD40^{-/-}$ mice (28, 30), suggest that APRIL, but not BAFF or CD40, is a major promoter of IgA production under physiologic conditions of antigen exposure.

A complex picture emerged from the analysis of IgA antibody responses in $APRIL^{-/-}$ mice, with the results depending on the route of immunization and the nature of the immunizing antigen. $APRIL^{-/-}$ mice had defective IgA antibody responses and markedly decreased numbers of IgA^+ plasma cells in the lamina propria of the small intestine after mucosal immunization with the TD antigen CGG. Again, this is unlikely to be due to disparity in genetic background between $APRIL^{-/-}$ and WT littermates because IgA antibody responses to mucosal immunization with CGG were comparable in the two parental strains (data not shown). The IgA antibody response of $APRIL^{-/-}$ mice to i.p.-introduced NP-CGG was equivalent to that of WT controls. However, they concomitantly mounted significantly increased IgG responses in all subclasses. In addition, $APRIL^{-/-}$ mice had a defective IgA antibody response to immunization with the TI-1 antigen NP-LPS but mounted a normal IgA antibody response to the TI-2 antigen NP-Ficoll. These results suggest that IgA antibody responses to mucosal immunization with protein antigens and to TI-1 antigen may preferentially involve APRIL, compared to i.p. immunization with TD antigen or TI-2 antigen. This is possibly because LPS from gut flora and in TI-1 antigens may preferentially induce APRIL, which drives IgA isotype switching. Because normally the majority of antigens are encountered via the mucosal route, deficiency in IgA antibody responses to mucosally introduced antigens in $APRIL^{-/-}$ mice may underlie their global serum IgA deficiency.

Both conventional B2 cells and B1 cells are implicated in the IgA response of the gut mucosa. IgA^+ cells of the B2 lineage are IL-6 dependent but IL-5-independent, but B1-derived IgA^+ cells are IL-5-dependent and IL-6-independent (40). Furthermore, intestinal IgA production induced by commensal bacteria is mainly performed by B2, not B1, cells (41). Further experiments are needed to determine whether both B1 and B2 cells are involved in the reduced IgA antibody response to oral immunization in $APRIL^{-/-}$ mice.

APRIL by itself was sufficient to drive isotype switching to IgA and IgG1 in IgM^+IgD^+ B cells derived from CD40-deficient mice, but required IL-4 for IgE isotype switching. This was not due to contamination by endotoxin, because the APRIL preparation contained <1 unit/mg protein, and because its effect was not inhibited by 5 μg/ml polymyxin B (data not shown). However, we cannot rule out the possibility that factors produced by B cells (or contaminating cells) synergized with BAFF/APRIL to induce

isotype switching. APRIL induced all of the molecular events known to be necessary for IgA isotype switching; namely, transcription at the Cα locus, expression of AID, Sμ-Sα deletional switch recombination, and expression of mature Cα transcripts. Furthermore, it also induced IgA secretion. BAFF and APRIL have been described to induce IgA isotype switching in human IgD^+ B cells, with BAFF directing switching to IgA1 and APRIL to IgA2 (42). However, unlike our results with mouse B cells, IgA protein was not detected in the supernatants of human B cells stimulated with APRIL or BAFF, unless they were also stimulated with anti-IgM and cytokines. This difference may be caused by species and/or technical differences.

The role of APRIL in physiological IgA switching demonstrated in this work raises the possibility that APRIL deficiency may underlie some cases of human IgA deficiency. It also suggests that APRIL may be useful as an adjuvant for vaccines delivered via the mucosal route.

We thank Drs. A. Kettner, S. Brodeur, and J. P. Manis for critical review of the manuscript and A. Flint and Q. Vu for excellent technical assistance. This work was supported by National Institutes of Health Grants AI31136 (to R.S.G.), AI31541 (to R.S.G.), DK47677 (to A.K.B.), and DK43351 (to A.K.B.), the Jeffrey Modell Foundation, Baxter Healthcare, and Mr. Neal Wallace.

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