

Transmembrane activator, calcium modulator, and cyclophilin ligand interactor drives plasma cell differentiation in LPS-activated B cells

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Background: Transmembrane activator, calcium modulator, and cyclophilin ligand interactor (TACI) expression on B cells is upregulated by Toll-like receptor (TLR) 4.

Objective: We sought to examine whether TACI synergizes with TLR4 in driving immunoglobulin production by B cells and to examine the mechanism of this synergy.

Methods: Purified mouse naive B cells were stimulated with the TACI ligand a proliferation-inducing ligand (APRIL) and with suboptimal concentrations of the TLR4 ligand LPS in the presence or absence of IL-4. Immunoglobulin secretion was measured by means of ELISA. Surface IgG1-positive B cells and CD138⁺ plasmacytoid cells were enumerated by means of FACS. Expression of $\gamma 1$ and ϵ germline transcripts, activation-induced cytidine deaminase, and $\gamma 1$ and ϵ mature transcripts was measured by means of RT-PCR.

Results: APRIL synergized with LPS in driving B-cell proliferation and IgM, IgG1, IgG3, IgE, and IgA production. This was mediated by TACI because it was preserved in B-cell maturation antigen^{-/-}, but not TACI^{-/-}, B cells. APRIL and LPS synergized to promote isotype switching, as evidenced by increased expression of activation-induced cytidine deaminase and $\gamma 1$ and ϵ mature transcripts and generation of surface IgG1-positive cells. More importantly, APRIL and LPS strongly synergized to drive the plasma cell differentiation program, as evidenced by an increase in CD138⁺ cells and expression of B lymphocyte induced maturation protein-1 (Blimp-1), interferon regulatory factor-4 (IRF-4), and the spliced form of X-box binding protein-1 (XBP-1). TACI^{-/-} mice had impaired IgM and IgG1 antibody responses to

immunization, with a suboptimal dose of the type I T cell-independent antigen 2, 4, 6- Trinitrophenol (TNP)-LPS.

Conclusions: These observations suggest that TACI cooperates with TLR4 to drive B-cell differentiation and immunoglobulin production *in vitro* and *in vivo*. (J Allergy Clin Immunol 2009;123:1000-1008.)

Key words: B cells, Toll-like receptor, LPS, transmembrane activator, calcium modulator, and cyclophilin ligand interactor, a proliferation-inducing ligand, immunoglobulin

The TNF superfamily members B cell-activating factor belonging to the TNF superfamily (BAFF) and a proliferation-inducing ligand (APRIL) play important roles in B-cell development, homeostasis, and activation. BAFF is expressed by neutrophils, monocytes, and dendritic cells (DCs) and promotes B-lymphocyte survival and maturation.¹ APRIL is expressed on monocytes/macrophages, DCs, and activated T cells.¹ APRIL^{-/-} mice have low serum IgA levels, impaired IgA antibody response to oral immunization, and decreased antibody response to the T cell-dependent antigen tetanus toxoid.^{2,3} BAFF and APRIL activate isotype switching in naive B cells, albeit modestly compared with CD40 or Toll-like receptor (TLR) ligation.^{4,5}

APRIL and BAFF share 2 receptors: transmembrane activator, calcium modulator, and cyclophilin ligand interactor (TACI) and B-cell maturation antigen (BCMA).⁶ TACI is expressed on a fraction of B cells.^{7,8} BCMA is poorly expressed on resting B cells but is upregulated on plasma cells and germinal center B cells.⁹ A third receptor, BAFF receptor, is unique for BAFF and is expressed mainly on B cells.¹⁰ APRIL also binds to heparan-sulfated proteoglycans (HSPGs).^{11,12} TACI^{-/-} mice have low serum IgM and IgA levels and have deficient antibody responses to the type II T cell-independent antigens Pneumovax (Merck & Co, Inc, Whitehouse Station, NJ) and TNP-Ficoll (Biosearch Technologies, Novato, Calif).¹³⁻¹⁵ TACI mediates isotype switching by APRIL.⁴ BCMA plays no detectable role in isotype switching.⁴

TLRs recognize specific molecular patterns of microbial pathogens¹⁶ and play an important role in the induction of adaptive immune responses, in part by promoting the maturation of antigen-presenting DCs to fully activate T and B cells.¹⁷ The TLR4 ligand LPS and the TLR9 ligand CpG activate isotype switching in B cells in the presence of cytokines.^{18,19} LPS drives class-switch recombination (CSR) to IgG3 on its own.²⁰

B cells encounter antigens in secondary lymphoid organs, where they proliferate and undergo maturation and differentiation that includes CSR, affinity maturation, and differentiation into

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Abbreviations used

AICDA:	Activation-induced cytidine deaminase
APRIL:	A proliferation-inducing ligand
BAFF:	B cell-activating factor belonging to the TNF superfamily
BCMA:	B-cell maturation antigen
Blimp-1:	B lymphocyte induced maturation protein-1
CSR:	Class-switch recombination
DC:	Dendritic cell
FITC:	Fluorescein isothiocyanate
GLT:	Germline transcript
HSPG:	Heparan-sulfated proteoglycan
IRF-4:	Interferon regulatory factor-4
NF:	Nuclear factor
PE:	Phycoerythrin
sIgG1:	Surface IgG1
TACI:	Transmembrane activator, calcium modulator, and cyclophilin ligand interactor
TLR:	Toll-like receptor
TNP:	2, 4, 6-Trinitrophenol
WT:	Wild-type
XBP-1:	X-box binding protein-1

plasma cells or memory B cells. These processes are initiated and regulated by signals derived from ligand-receptor interactions that include CD40 ligand-CD40, BAFF/APRIL-TACI, and TLR ligand-TLR interactions. The CD40 ligand-CD40 interaction occurs in germinal centers and involves follicular T_H cells and their expression of inducible costimulator and IL-21.²¹ CD40 ligation upregulates TACI expression and synergizes with TACI ligation to enhance immunoglobulin production by naive B cells.²² The TLR ligands TLR4 and TLR9 also upregulate TACI expression, and TLR9 synergizes with TACI to increase immunoglobulin production by B cells.¹⁸ In this study we show that APRIL synergizes with suboptimal doses of LPS in driving naive mouse B cells to proliferate, generate plasmacytoid cells, and secrete immunoglobulins *in vitro*. We also provide evidence for synergy between TACI and TLR4 in the *in vivo* antibody response to the prototypic T cell-independent type I antigen TNP-LPS, which focuses LPS on TNP-specific B cells, resulting in their activation and differentiation through TLR-4-mediated signaling in a T cell-independent manner.

METHODS**Mice**

BALB/c mice were purchased from Charles River Laboratories (Wilmington, Mass). TACI^{-/-}, BCMA^{-/-}, and genetically matched wild-type (WT) mice on a Sv129xC57Bl6 background were previously described.^{14,23} All mice were bred and housed in a specific pathogen-free animal facility. All experimental procedures performed on the animals were approved by the Animal Care and Use Committee of the Children's Hospital, Boston, Massachusetts.

Antibodies and flow cytometric analysis

B cells were stained with anti-TACI-phycoerythrin (PE), anti-BCMA-fluorescein isothiocyanate (FITC), or anti-BAFF receptor-FITC (R&D Systems, Minneapolis, Minn) and anti-B220-FITC, anti-CD138-PE, or anti-IgG1-PE (BD Pharmingen, San Jose, Calif). For survival assays, B cells were stained with Annexin V-FITC and propidium iodide (BioVision, Mountain View, Calif). Stained cells were analyzed by means of FACS (BD FACSscan).

Proliferation and *in vitro* immunoglobulin production

Naive B cells were negatively sorted from mouse splenocytes; cultured with APRIL (1 µg/mL, R&D Systems), IL-4 (50 ng/mL, R&D Systems), and LPS (*Escherichia coli* 026:B6; Sigma, St Louis, Mo); and assayed for proliferation and immunoglobulin production, as previously described.²⁴

RT-PCR analysis

RNA extraction from 4-day cultures and PCR conditions used to detect Iε-Cε, Iµ-Cε, Iγ1-Cγ1, Iµ-Cγ1, AICDA, and β₂-microglobulin were previously described.²⁵

Q-PCR analysis

Real-time PCR reactions were run on cDNA by using ABI Prism 7300 (Applied Biosystems, Foster City, Calif), as detailed in the Methods section of this article's Online Repository at www.jacionline.org.

***In vivo* antibody response to TNP-LPS**

Genetically matched WT and TACI^{-/-} mice were immunized intraperitoneally with a single injection of 10 µg per mouse of TNP(.4)-LPS (Biosearch Technologies). Sera were collected at day 14 after immunization, and serial dilutions were analyzed for TNP-specific IgM, IgA, IgG1, and IgG3 antibodies by means of ELISA.

Statistics

P values were calculated with the paired *t* test for *in vitro* data and 2-way ANOVA for *in vivo* data by using PRISM software (Prism Software Corp, Irvine, Calif).

RESULTS**APRIL enhances LPS-driven immunoglobulin production in naive B cells**

Initial experiments in which naive B cells (95% B220⁺IgM⁺IgD⁺ cells) were stimulated with a standard concentration of 10 µg/mL LPS did not reveal an enhancing effect of APRIL on immunoglobulin production (data not shown). We therefore examined the effect of APRIL on B cells stimulated with a suboptimal concentration of 100 ng/mL LPS. This concentration was selected based on pilot experiments, in which a range of LPS concentrations (50 ng/mL to 10 µg/mL) were tested for their ability to drive IgG1 and IgE synthesis in the presence of IL-4 (see Fig E1, A, in this article's Online Repository at www.jacionline.org). Relatively weak induction of proliferation and immunoglobulin production has been previously documented with 1 µg/mL APRIL compared with anti-CD40 and LPS.⁴ There was only a modest difference (<2.5-fold change) between the effects of different APRIL concentrations tested (range, 50 ng/mL to 4 µg/mL) on B-cell proliferation and production of IgG1, IgE, and IgA (see Table E1 in this article's Online Repository at www.jacionline.org). Fig 1, A, shows that APRIL (1 µg/mL) significantly enhanced IgM (approximately 6-fold) and IgA (approximately 2.7-fold) secretion in B cells stimulated with 100 ng/mL LPS to a level comparable with that induced by 10 µg/mL LPS. APRIL also significantly enhanced IgG1 and IgE synthesis driven by 100 ng/mL LPS plus IL-4 (approximately 2.5-fold and 6-fold, respectively) to levels comparable with those secreted by B cells stimulated with 10 µg/mL LPS plus IL-4 (Fig 1, B). Note that APRIL plus IL-4 induced weak, but detectable, IgG1 (24 ± 10 ng/mL, *n* = 3) and IgE (6 ± 3 ng/mL, *n* = 3) secretion, as previously described.⁶

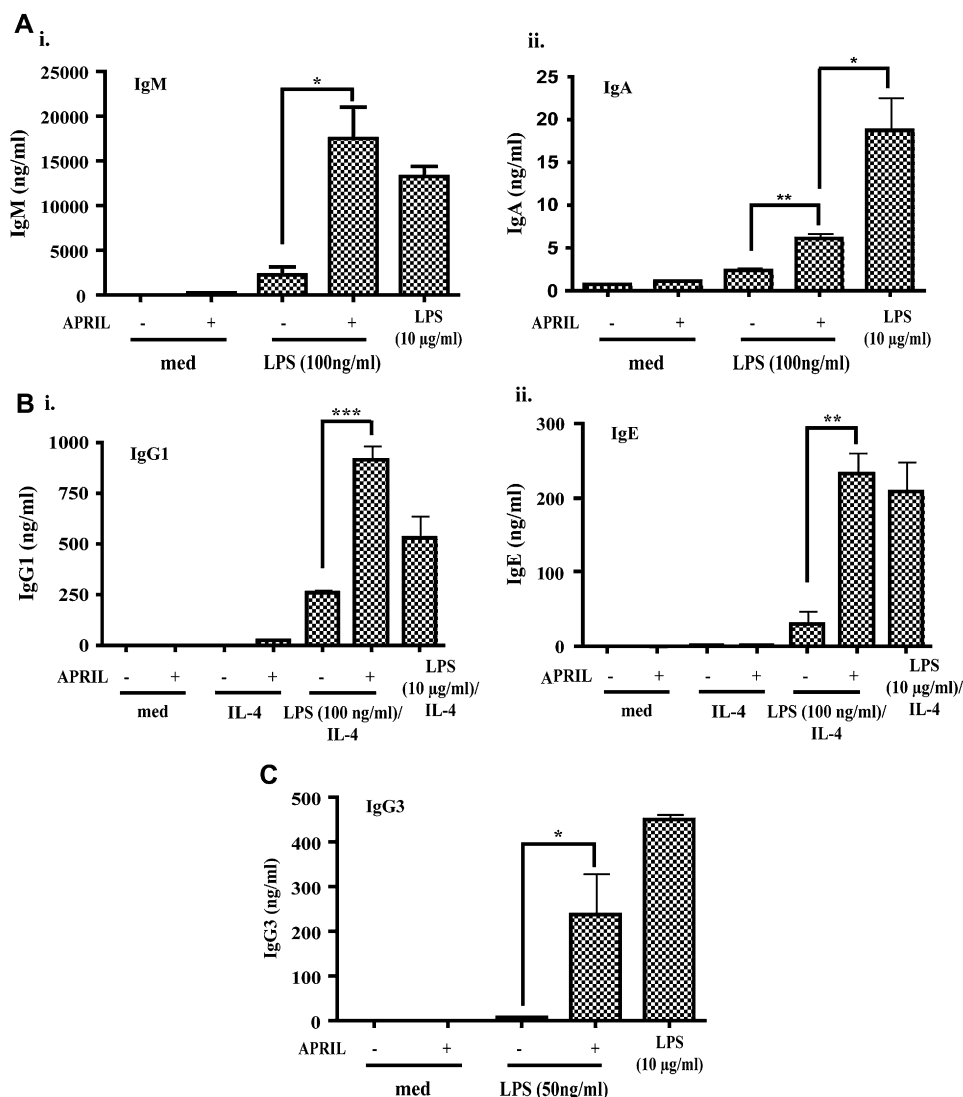


FIG 1. APRIL enhances LPS-driven immunoglobulin production in naive B cells. The effect of APRIL (1 µg/mL) on IgM (A, i), IgA (A, ii), IgG1 (B, i), IgE (B, ii), and IgG3 (C) production by B cells stimulated with sub-optimal concentrations of LPS is shown. The *right-hand bars* depict the amounts of immunoglobulins secreted in cultures stimulated with 10 µg/mL LPS. Values represent the mean $\pm$ SD of at least 3 experiments. * $P < .05$, ** $P < .01$, and *** $P < 0.001$. med, Medium.

LPS is a potent inducer of IgG3 synthesis.²⁰ Based on pilot experiments (see Fig E1, B, in this article's Online Repository), we chose an LPS concentration of 50 ng/mL as the suboptimal LPS concentration to examine synergy with APRIL in inducing IgG3 synthesis. Fig 1, C, shows that APRIL significantly enhanced IgG3 secretion by B cells stimulated with 50 ng/mL LPS (approximately 25-fold) to a level comparable with that obtained with 10 µg/mL LPS. Taken together, these results indicate that APRIL synergizes with LPS to drive immunoglobulin synthesis.

APRIL enhancement of LPS-driven immunoglobulin production is dependent on TACI

APRIL engages TACI, BCMA, and HSPGs. To elucidate which of these receptors mediates the enhancing effect of APRIL on LPS-driven immunoglobulin production, we examined the effect of APRIL on LPS-induced immunoglobulin production in B cells from TACI^{-/-} mice, BCMA^{-/-} mice, and WT control animals on

the same Sv129xC57Bl6 background. Fig 2 shows that APRIL significantly enhanced LPS-driven IgM, IgA, IgG1, and IgE secretion by B cells from BCMA^{-/-} mice and WT control animals but not secretion by B cells from TACI^{-/-} mice. Variations in absolute amounts of immunoglobulins secreted by B cells from the 3 groups of mice studied, including higher IgG1 production by TACI^{-/-} mice compared with that seen in WT control animals, likely reflect their mixed Sv129xC57Bl6 background, the effect of increased stimulation by intestinal flora in an IgA-deficient host in the case of increased IgG1 production in TACI^{-/-} mice, or both. These results indicate that TACI mediates the synergy of APRIL with LPS.

APRIL and LPS synergize to increase B-cell proliferation and survival

CSR and subsequent immunoglobulin secretion are thought to require B-cell proliferation.²⁶ We tested the effect of APRIL on LPS-driven proliferation of B cells from BALB/c WT mice by

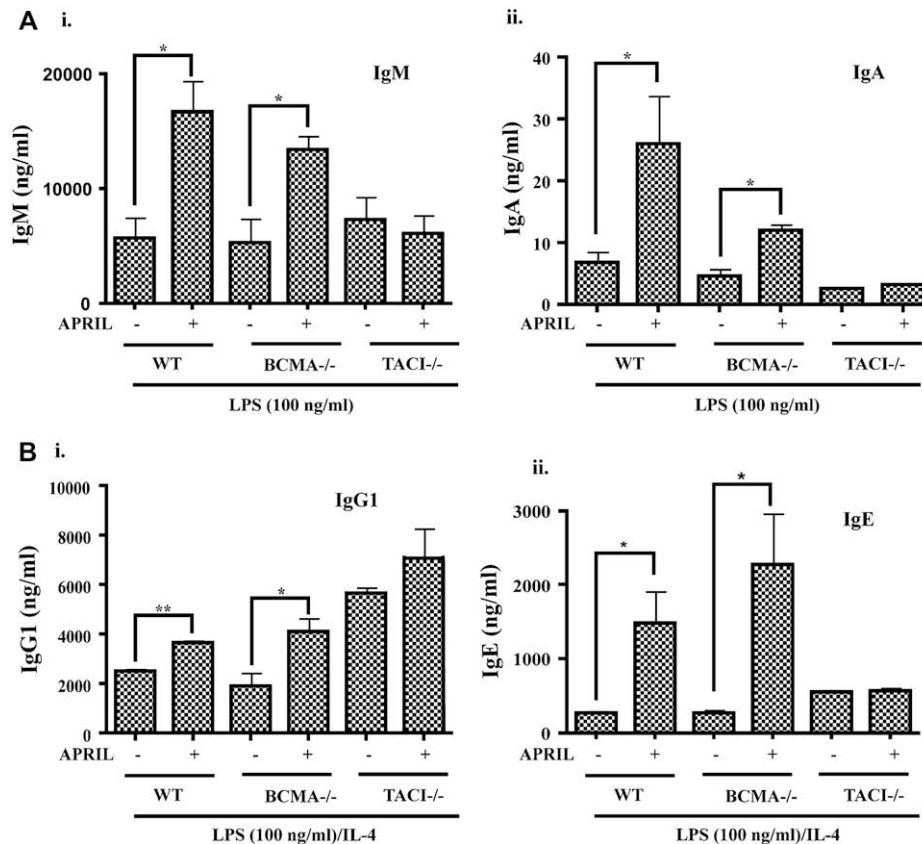


FIG 2. APRIL enhancement of LPS-driven immunoglobulin production is dependent on TACI. The effect of APRIL (1 μ g/mL) on IgM (A, i), IgA (A, ii), IgG1 (B, i), and IgE (B, ii) production by naive B cells from TACI^{-/-} mice, BCMA^{-/-} mice, and genetically matched WT control animals is shown. Values represent the mean $\pm$ SD of at least 3 experiments. * P < .05 and ** P < .01.

examining tritiated thymidine incorporation after 3 days in culture. Fig 3, A, shows that APRIL enhanced the proliferation of naive B cells from WT BALB/c mice stimulated with 100 ng/mL LPS, both in the presence and absence of IL-4. APRIL caused an approximately 3.5-fold increase in tritiated thymidine incorporation in LPS-stimulated cultures and an approximately 5-fold increase in LPS plus IL-4-stimulated cultures. However, this proliferation remained significantly lower than that caused by 10 μ g/mL LPS. APRIL caused a significant increase in the number of live cells in LPS-stimulated cultures after 4 days of stimulation, which averaged 3.0 ± 1.1 -fold in the absence of IL-4 and 1.9 ± 0.4 -fold in the presence of IL-4 (data not shown). The effect of APRIL on B-cell proliferation was mediated by TACI because APRIL enhanced LPS- and LPS/IL-4-driven proliferation of B cells from BCMA^{-/-} mice but not from TACI^{-/-} mice (Fig 3, B). Variations in the absolute amount of tritiated thymidine incorporated into DNA of B cells from the 3 groups of mice studied likely reflect their mixed Sv129xC57B16 background.

We examined the effect of APRIL on the survival of B cells stimulated with LPS. We stained cells 3 days after stimulation with Annexin V and propidium iodide to identify live cells (Annexin V⁻, propidium iodide-negative cells). Fig 3, C, is a representative FACS analysis and shows that the percentage of live cells was substantially higher among cells stimulated with APRIL plus LPS (100 ng/mL) compared with that seen among cells stimulated only with LPS (100 ng/mL), both in the absence and

presence of IL-4. Pooled results from 3 experiments demonstrated that APRIL significantly increased the percentage of live cells in LPS plus IL-4-stimulated cultures (Fig 3, D). The percentage of live B cells in LPS (100 ng/mL)-stimulated cultures was $7.0\% \pm 1.8\%$, and it increased to $15.8\% \pm 0.7\%$ with the addition of APRIL. The percentage of live B cells in LPS (100 ng/mL) plus IL-4-stimulated cultures was $30.9\% \pm 4.8\%$, and it increased to $44.1\% \pm 4.9\%$ with the addition of APRIL.

Increased B-cell proliferation and survival might contribute to APRIL's enhancement of LPS-driven immunoglobulin production.

APRIL and LPS synergize in driving CSR

To determine whether APRIL synergizes with LPS in causing CSR, we first examined surface IgG1 (sIgG1) expression in naive B cells stimulated with LPS plus IL-4 for 6 days in the presence or absence of APRIL. Fig 4, A, shows that there were virtually no detectable sIgG1⁺ B cells in the starting population of sorted naive B cells. APRIL plus IL-4 induced a small but detectable increase in sIgG1 expression. Stimulation with 100 ng/mL LPS plus IL-4 induced expression of sIgG1. Addition of APRIL to these cultures increased the percentage of sIgG1⁺ B cells. Pooled results from 3 experiments demonstrated that APRIL significantly increased the percentage of sIgG1⁺ cells (approximately 2-fold) in cultures stimulated with 100 ng/mL LPS plus IL-4. This percentage

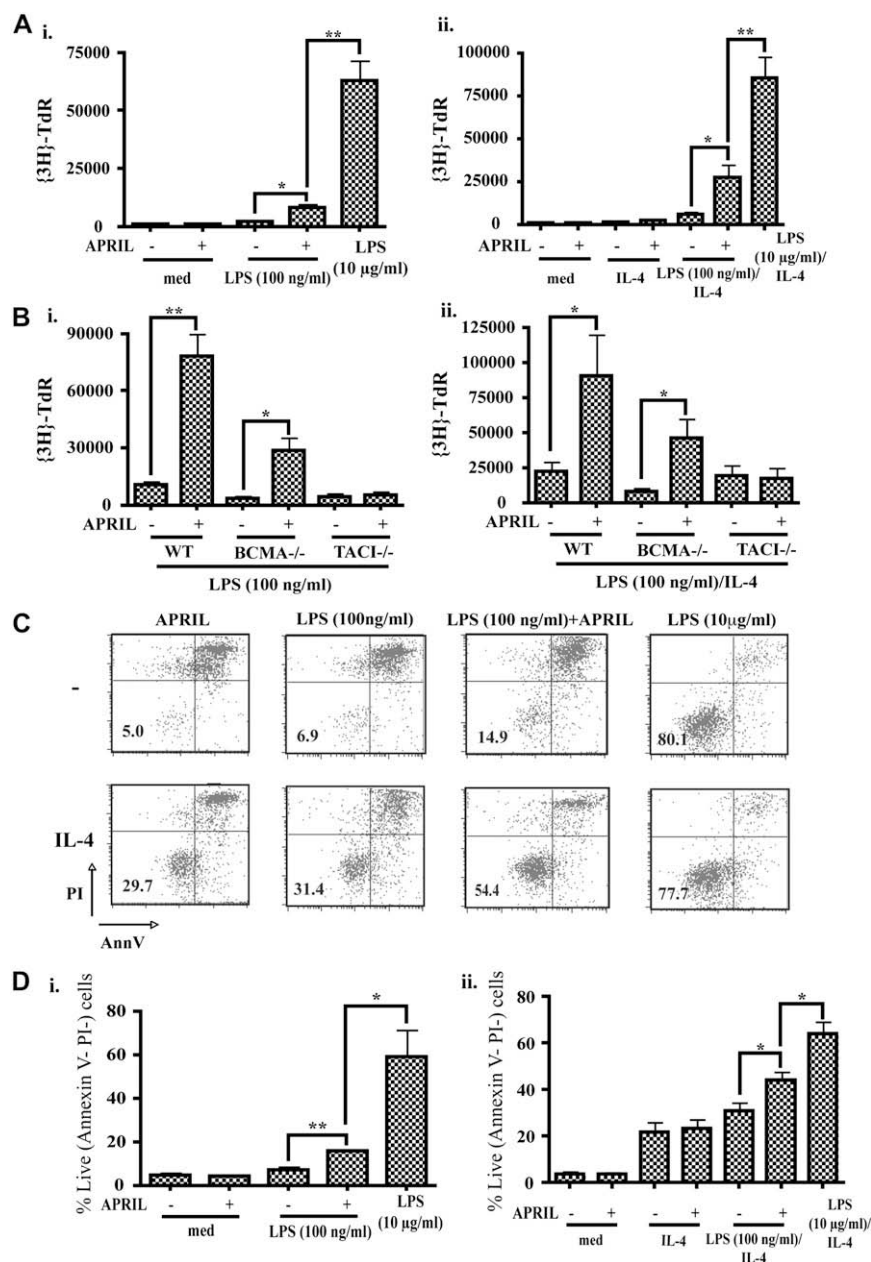


FIG 3. APRIL and LPS synergize to increase proliferation and survival of naive B cells. **A**, Effect of APRIL on LPS-driven proliferation of B cells in cultures without IL-4 (*i*) and with IL-4 (*ii*). *med*, Medium. **B**, Effect of APRIL on the proliferation of B cells from TACI^{-/-}, BCMA^{-/-}, and WT mice in cultures without IL-4 (*i*) and with IL-4 (*ii*). [³H]-TdR, Tritiated thymidine. **C**, FACS analysis of Annexin V (AnnV) and propidium iodide staining of B cells cultured for 3 days. **D**, Pooled results of 3 survival experiments. **P* < .05 and ***P* < .01.

was, however, significantly lower than that achieved with 10 µg/mL LPS plus IL-4 (Fig 4, A). The percentages of sIgG1⁺ cells are net percentages obtained after subtracting the percentage of cells that were stained by the isotype controls, which in all cases was less than 1.5%.

Molecular events involved in CSR include expression of germline transcripts (GLTs) and *AICDA*, followed by deletional switch recombination and expression of Iµ-C_H mature transcripts.²⁷ We tested the effect of APRIL on molecular events involved in CSR to IgE and IgG1 driven by 100 ng/mL LPS plus IL-4. Fig 4, B, shows that, as previously reported, IL-4 alone does not induce CεGLT in naive mouse B cells.⁴ CεGLT was

weakly induced by APRIL plus IL-4. CεGLT induction by LPS plus IL-4 was stronger and was slightly enhanced by APRIL. Cε1GLT was induced by IL-4 but was not consistently upregulated by addition of APRIL, 100 ng/mL LPS, or both. APRIL plus IL-4 induced *AICDA*, as previously described.⁴ APRIL clearly synergized with 100 ng/mL LPS plus IL-4 in inducing *AICDA*, mature Iµ-Cε transcripts, and mature Iµ-Cγ1 transcripts. Expression of Iµ-Cγ1 transcripts in APRIL plus IL-4-stimulated B cells was only detected with an increased number of cycles of RT-PCR amplification (data not shown). Taken together, the above data suggest that APRIL synergizes with LPS in causing CSR.

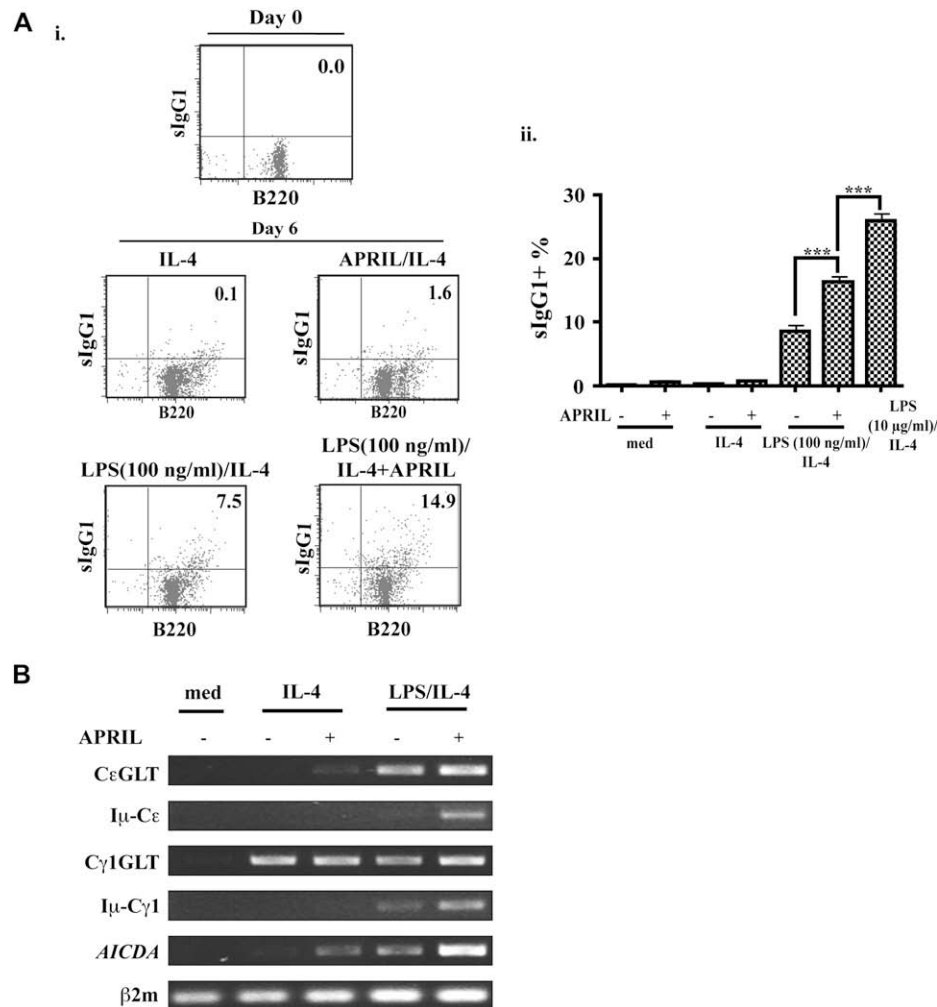


FIG 4. APRIL and LPS synergize to increase sIgG1 expression and to induce molecular events involved in CSR in naive B cells. **A**, Representative FACS analysis (*i*) and pooled results of 3 experiments (*ii*) of sIgG1 expression in B cells stimulated for 6 days. * $P < .05$, ** $P < .01$, and *** $P < .001$. **B**, Expression of CεGLT, Iµ-Cε, Cγ1GLT, Iµ-Cγ1, and AICDA mRNA by means of RT-PCR in B cells stimulated for 4 days. Similar results were obtained in 3 independent experiments.

APRIL strongly synergizes with LPS to induce plasma cell differentiation

Plasma cells secrete immunoglobulins at a high rate. We investigated whether APRIL synergizes with LPS in the generation of cells that express syndecan-1/CD138, a marker for plasmacytoid cells. Fig 5, A, shows that there were few CD138⁺ cells in cultures stimulated with APRIL, APRIL plus IL-4, or 100 ng/mL LPS plus IL-4. Addition of APRIL markedly increased (by approximately 4-fold) the percentage of CD138⁺ cells in cultures stimulated with 100 ng/mL LPS plus IL-4 to a level significantly higher than that induced by 10 µg/mL LPS plus IL-4. APRIL also significantly increased the percentage of CD138⁺ cells in cultures stimulated with 100 ng/mL LPS alone by approximately 2.5-fold (data not shown). The percentages shown are net percentages of positive cells.

Differentiation of B cells into CD138⁺ plasma cells correlates with increased expression of the transcription factors Blimp-1 and IRF-4.²⁸ We measured mRNA levels for Blimp-1 and IRF-4 in IL-4-stimulated cultures by means of Q-PCR. Fig 5, B, shows that LPS (100 ng/mL) induced a 2.5-fold increase in Blimp-

1 mRNA levels, whereas APRIL had no detectable effect. However, APRIL synergized with LPS (100 ng/mL) to cause a significant increase (approximately 3-fold) in Blimp-1 mRNA expression and, to a lesser extent, in IRF-4 mRNA expression. Blimp-1 and IRF-4 synergize to induce XBP-1 splicing and plasma cell differentiation.²⁸ Fig 5, B, shows that APRIL synergized with LPS (100 ng/mL) plus IL-4 to induce a significant increase (2.5-fold) in the expression of XBP-1(s) (the spliced form of XBP-1). Taken together, these results indicate that APRIL strongly drives the plasma cell differentiation program in LPS-stimulated B cells.

APRIL was recently shown to induce Bcl-X_L expression in plasmacytoid cells and to be important for their survival *in vivo*.³ Mcl-1 has also been reported to be induced by APRIL and to be important for plasma cell survival.^{12,29} Addition of APRIL to LPS-stimulated B cells had no detectable effect on Bcl-X_L expression by CD138⁺ cells, as determined by means of intracellular staining (see Fig E2 in this article's Online Repository at www.jacionline.org) or on Bcl-X_L and Mcl-1 mRNA expression, as determined by means of Q-PCR (data not shown).

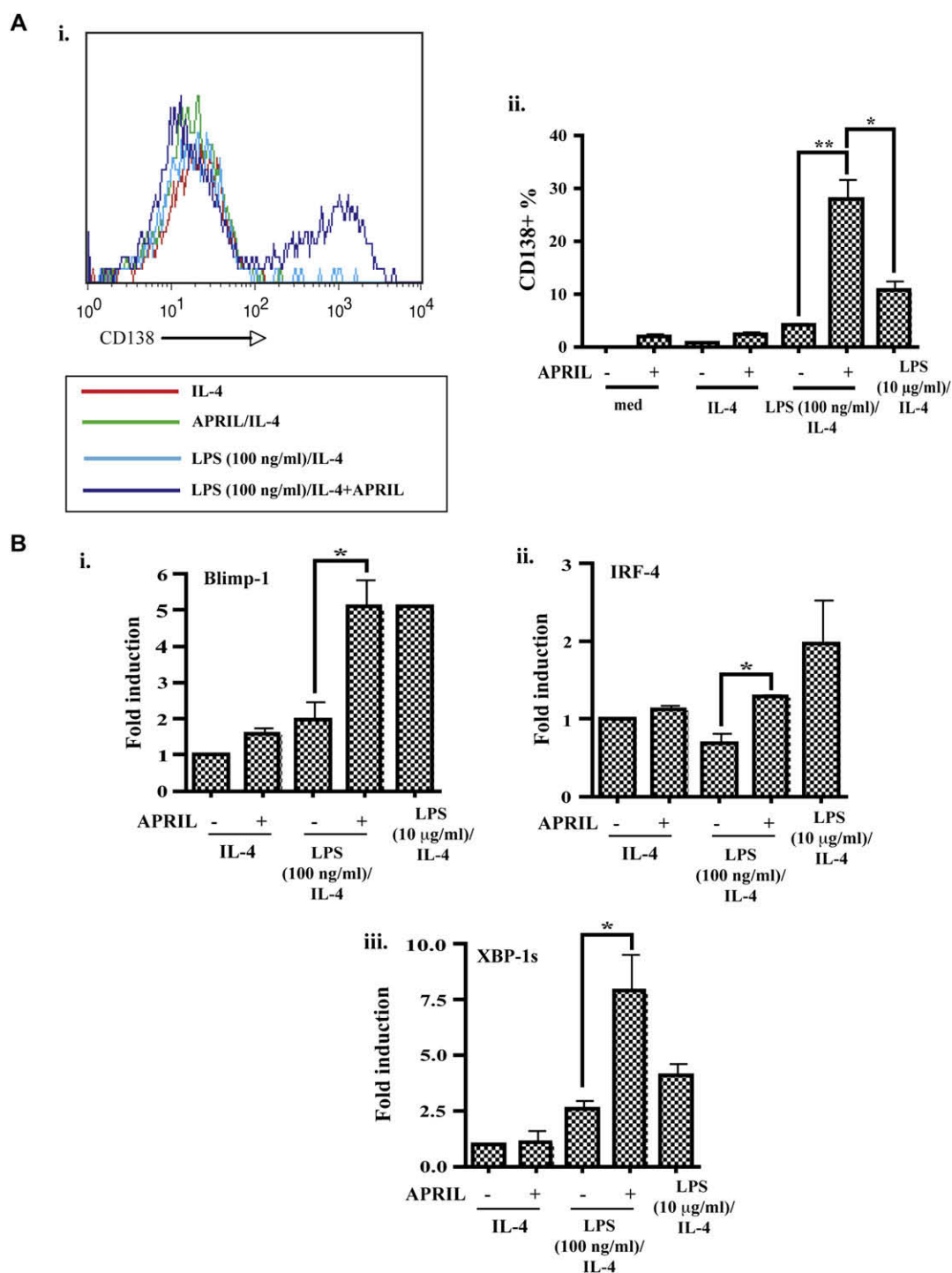


FIG 5. APRIL synergizes with LPS plus IL-4 to increase the surface CD138 expression and to induce mRNA expression of Blimp-1, IRF-4, and the spliced form of XBP-1. **A**, Representative FACS analysis (*i*) and pooled results of 3 experiments (*ii*) of surface CD138 expression in B cells stimulated for 6 days. *med*, Medium. **B**, Q-PCR analysis of mRNA levels for Blimp-1 (*i*), IRF-4 (*ii*), and XBP-1(s) (*iii*) in B cells. * $P < .05$, ** $P < .01$, and *** $P < .001$.

TACI-deficient mice have impaired antibody responses to immunization with a suboptimal dose of TNP-LPS

Antigen-specific B cells responding to the T cell-independent antigen TNP-LPS *in vivo* receive signals through TLR4 and

potentially through TACI from DC-derived BAFF/APRIL. We reasoned that if TACI ligation synergizes with TLR4 in driving the response of B cells to type I T cell-independent antigens *in vivo*, TACI^{-/-} mice could exhibit an impaired antibody response to a suboptimal dose of TNP-LPS. Consistent with published data,¹³

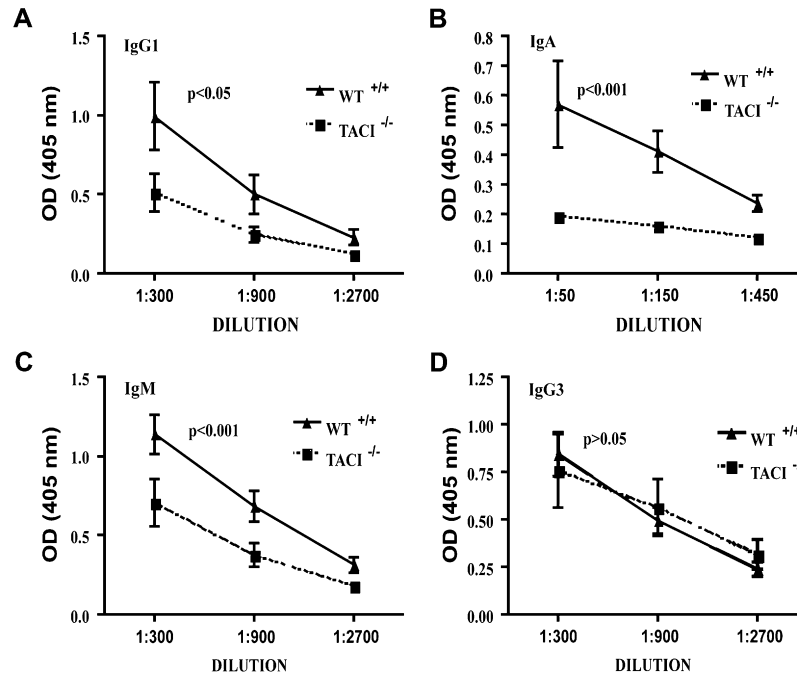


FIG 6. Impaired antibody response to immunization with low-dose TNP-LPS (10 μ g per mouse) in TACI $^{-/-}$ mice. IgG1 (A), IgA (B), IgM (C), and IgG3 (D) anti-TNP antibody levels of TACI $^{-/-}$ and WT mice are shown. Results represent the mean $\pm$ SE of 9 mice. Dilution curves were compared by means of ANOVA.

initial experiments revealed that TACI $^{-/-}$ mice and WT control animals mounted comparable antibody responses to immunization with a dose of 50 μ g of TNP-LPS (data not shown). We then examined the response of these mice to a suboptimal dose of 10 μ g of TNP-LPS per mouse. Fig 6 shows that TACI $^{-/-}$ mice had impaired IgM, IgA, and IgG1 antibody responses to 10 μ g of TNP-LPS compared with those seen in WT control animals. TACI $^{-/-}$ mice immunized with 10 μ g of TNP-LPS plus 40 μ g of LPS similarly showed a significant defect in TNP-specific IgM, IgA, and IgG1 antibody responses compared with those seen in WT mice (see Fig E3 in this article's Online Repository at www.jacionline.org). This suggests that TNP and LPS need to be physically linked for an optimal antibody response, possibly because linking TNP to LPS focuses the LPS signal on TNP-specific B cells. There was no impairment of the IgG3 antibody response to this dose in TACI $^{-/-}$ mice. These results suggest that TACI synergizes with TLR4 in driving the antibody response to suboptimal amounts of type I T cell-independent antigen *in vivo*.

DISCUSSION

We demonstrate that TACI synergizes with TLR4 in enhancing immunoglobulin production *in vitro* and promotes the antibody response to T cell-independent type I antigen *in vivo*.

APRIL synergized with suboptimal concentrations of LPS to enhance the production of all immunoglobulin isotypes examined, which included IgM, IgA, IgG3, IgG1, and IgE (Fig 1). For all these isotypes, the amounts of immunoglobulin secreted in response to a suboptimal concentration of LPS plus APRIL were comparable with those induced by the maximal concentration of 10 μ g/mL LPS. There is both indirect and direct evidence that the effect of APRIL on LPS-driven immunoglobulin production was mediated through TACI. BCMA is poorly expressed, if at all, by splenic B cells and is not upregulated by LPS (our

unpublished observations).¹⁸ More importantly, APRIL enhanced LPS-driven immunoglobulin production in BCMA-deficient B cells but not in TACI-deficient B cells (Fig 2). These observations argue against a role for BCMA or HSPGs and for an exclusive role of TACI in the synergistic effect of APRIL on LPS-driven immunoglobulin production by naive B cells.

APRIL synergized with LPS in inducing B-cell proliferation and survival, which might have contributed to increased immunoglobulin production. The ability of APRIL to enhance the survival of primary murine B cells activated by LPS is consistent with its ability to promote increased viability of human B cells stimulated with anti-IgM antibodies¹² and of chronic lymphocytic leukemia B cells³⁰ and is consistent with its antiapoptotic effect on malignant human B cells.³¹⁻³³ CSR induced by LPS is dependent on proliferation.³⁴ Given this fact, it is likely that B-cell proliferation is necessary for enhanced immunoglobulin secretion by LPS-stimulated B cells on addition of APRIL.

APRIL promoted isotype switching induced by LPS plus IL-4, as evidenced by a significant increase in the percentage and numbers of sIgG1 $^{+}$ cells (Fig 4, A). APRIL had a minimal effect on the expression of C ϵ and C γ 1 GLTs induced by LPS plus IL-4. However, it strongly enhanced the expression of AICDA and of mature postswitch I μ -C ϵ and I μ -C γ 1 transcripts (Fig 4, B). These results suggest that APRIL enhances LPS-driven CSR by upregulating AICDA expression. An additional effect on the viability of switched B cells is possible.

The most striking effect of the addition of APRIL to B-cell cultures stimulated with a suboptimal concentration of LPS was an increase in the percentage of CD138 $^{+}$ cells that significantly exceeded the percentage observed in cultures stimulated with optimal concentrations of LPS (Fig 5, A). The fact that B cells that differentiate more vigorously into plasmacytoid cells have less time to proliferate might explain the observation that generation of CD138 $^{+}$ cells was more potently enhanced than proliferation through

addition of APRIL to LPS-stimulated B cells. Because CD138⁺ cells produce large amounts of immunoglobulins compared with CD138⁻ B cells, the increase in CD138⁺ cells probably accounts for most of the increased immunoglobulin production in B-cell cultures stimulated with APRIL and LPS. Consistent with the increased percentages of CD138⁺ cells, APRIL synergized with LPS (100 ng/mL) in inducing Blimp-1 and, to a lesser extent, IRF-4 and in inducing increased expression of the spliced form of XBP-1 (Fig 5, B). Thus APRIL synergized with LPS in activating the plasma cell differentiation program. The increase in plasmacytoid cells was not accompanied by a detectable increase in the expression of Bcl-X_L (see Fig E2) or Mcl-1 (data not shown), 2 factors that are important for plasma cell survival.

Synergy between TACI and TLR4 was demonstrated *in vivo* by the observation that TACI^{-/-} mice on the Sv129xC57Bl6 background, which were used for all *in vitro* experiments, have impaired IgM, IgA, and IgG1 antibody responses to immunization with 10 µg of TNP-LPS (Fig 6), a dose 5-fold lower than the usual standard dose of 50 µg. These results were confirmed in TACI^{-/-} mice on a C57Bl6 background, suggesting that TACI might be required for an optimal response to type I T cell-independent antigen. The equivalent dose of 10 µg of TNP-LPS dose per mouse is 35 mg for a 70-kg man, a substantial dose of immunogen. The observation that the IgG3 response of TACI^{-/-} mice to 10 µg of TNP-LPS was unaffected simply reflects the higher sensitivity of IgG3 isotype switching to LPS. Immunization of TACI^{-/-} mice with doses of TNP-LPS less than 10 µg might uncover synergy between TACI and TLR4 in the *in vivo* IgG3 response to type I T cell-independent antigen.

The present work shows that in addition to synergizing with CD40 and TLR9, TACI ligation synergizes with TLR4 in driving immunoglobulin production by B cells.^{18,22} Upregulation of TACI has been invoked in the synergy of APRIL with CD40 and TLR9 in driving immunoglobulin production. Similarly, TACI upregulation by TLR4 might contribute to the synergy of APRIL with LPS.^{18,35} The mechanism by which TACI ligation enhances B-cell activation by LPS remains to be determined. TACI has been reported to activate the canonical nuclear factor (NF) κB pathway in B-cell lines.^{30,32} We were unable to detect NF-κB activation in primary B cells treated with APRIL, as assessed based on IκB phosphorylation, IκB degradation, and p65 translocation in a time course that spanned 5 minutes to 12 hours (data not shown). Furthermore, addition of APRIL to LPS (100 ng/mL) caused no significant enhancement of p65 nuclear translocation. In addition, APRIL and LPS, alone or in combination, caused no detectable activation of the noncanonical NF-κB pathway, as measured by nuclear appearance of p52 (data not shown). TACI activates nuclear factor of activated T cells through its interaction with Calcium-signal modulating ligand (CAML).³⁶ It is possible that activation of the CAML–nuclear factor of activated T cells (NF-AT) pathway by TACI might contribute to its synergy with LPS.

Our data indicate that in addition to its ability to trigger isotype switching,^{4,37} TACI plays an important role in plasma cell differentiation of B cells. Furthermore, TACI was shown to be important for the antibody response to suboptimal doses of type I antigen. It remains to be established whether TACI promotes the differentiation of TLR-stimulated human B cells into plasma cells. Impairment of TACI-driven plasmacytoid cell generation and immunoglobulin production by B cells stimulated with the TLR ligands might contribute to the low serum immunoglobulin levels seen in patients with common variable immunodeficiency with TACI mutations.

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Clinical implications: Impaired synergy of TACI with TLRs might contribute to low serum immunoglobulin levels in patients with common variable immunodeficiency with TACI mutations.

REFERENCES

- Mackay F, Ambrose C. The TNF family members BAFF and APRIL: the growing complexity. *Cytokine Growth Factor Rev* 2003;14:311-24.
- Castigli E, Scott S, Dedeoglu F, Bryce P, Jabara H, Bhan AK, et al. Impaired IgA class switching in APRIL-deficient mice. *Proc Natl Acad Sci U S A* 2004;101:3903-8.
- Belnoue E, Pihlgren M, McGaha TL, Tougne C, Rochat A, Bossen C, et al. APRIL is critical for plasmablast survival in the bone marrow and poorly expressed by early life bone marrow stromal cells. *Blood* 2008;111:2755-64.
- Castigli E, Wilson SA, Scott S, Dedeoglu F, Xu S, Lam KP, et al. TACI and BAFF-R mediate isotype switching in B cells. *J Exp Med* 2005;201:35-9.
- Litinskiy MB, Nardelli B, Hilbert DM, He B, Schaffer A, Casali P, et al. DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. *Nat Immunol* 2002;3:822-9.
- Mackay F, Kallied SL. TNF ligands and receptors in autoimmunity: an update. *Curr Opin Immunol* 2002;14:783-90.
- Wang H, Marsters SA, Baker T, Chan B, Lee WP, Fu L, et al. TACI-ligand interactions are required for T cell activation and collagen-induced arthritis in mice. *Nat Immunol* 2001;2:632-7.
- Seyler TM, Park YW, Takemura S, Bram RJ, Kurtin PJ, Goronzy JJ, et al. BLyS and APRIL in rheumatoid arthritis. *J Clin Invest* 2005;115:3083-92.
- Zhang X, Park CS, Yoon SO, Li L, Hsu YM, Ambrose C, et al. BAFF supports human B cell differentiation in the lymphoid follicles through distinct receptors. *Int Immunol* 2005;17:779-88.
- Mackay F, Schneider P, Rennert P, Browning J. BAFF AND APRIL: a tutorial on B cell survival. *Annu Rev Immunol* 2003;21:231-64.
- Ingold K, Zumsteg A, Tardivel A, Huard B, Steiner QG, Cachero TG, et al. Identification of proteoglycans as the APRIL-specific binding partners. *J Exp Med* 2005;201:1375-83.
- Mongini PK, Inman JK, Han H, Fattah RJ, Abramson SB, Attur M. APRIL and BAFF promote increased viability of replicating human B2 cells via mechanism involving cyclooxygenase 2. *J Immunol* 2006;176:6736-51.
- von Bulow GU, van Deursen JM, Bram RJ. Regulation of the T-independent humoral response by TACI. *Immunity* 2001;14:573-82.
- Yan M, Wang H, Chan B, Roose-Girma M, Erickson S, Baker T, et al. Activation and accumulation of B cells in TACI-deficient mice. *Nat Immunol* 2001;2:638-43.
- Seshasayee D, Valdez P, Yan M, Dixit VM, Tumas D, Grewal IS. Loss of TACI causes fatal lymphoproliferation and autoimmunity, establishing TACI as an inhibitory BLyS receptor. *Immunity* 2003;18:279-88.
- Kawai T, Akira S. Pathogen recognition with Toll-like receptors. *Curr Opin Immunol* 2005;17:338-44.
- Reis e Sousa C, Diebold SD, Edwards AD, Rogers N, Schulz O, et al. Regulation of dendritic cell function by microbial stimuli. *Pathol Biol (Paris)* 2003;51:67-8.
- Katsenelson N, Kanswal S, Puig M, Mostowski H, Verthelyi D, Akkoyunlu M. Synthetic CpG oligodeoxynucleotides augment BAFF- and APRIL-mediated immunoglobulin secretion. *Eur J Immunol* 2007;37:1785-95.
- Phipps RP, Roper RL, Stein SH. Regulation of B-cell tolerance and triggering by macrophages and lymphoid dendritic cells. *Immunol Rev* 1990;117:135-58.
- Rothman P, Lutzker S, Gorham B, Stewart V, Coffman R, Alt FW. Structure and expression of germline immunoglobulin gamma 3 heavy chain gene transcripts: implications for mitogen and lymphokine directed class-switching. *Int Immunol* 1990;2:621-7.
- King C, Tangye SG, Mackay CR. T follicular helper (TFH) cells in normal and dysregulated immune responses. *Annu Rev Immunol* 2008;26:741-66.
- Castigli E, Wilson SA, Elkhail A, Ozcan E, Garibyan L, Geha RS. Transmembrane activator and calcium modulator and cyclophilin ligand interactor enhances CD40-driven plasma cell differentiation. *J Allergy Clin Immunol* 2007;120:885-91.
- Xu S, Lam KP. B-cell maturation protein, which binds the tumor necrosis factor family members BAFF and APRIL, is dispensable for humoral immune responses. *Mol Cell Biol* 2001;21:4067-74.

24. Castigli E, Alt FW, Davidson L, Bottaro A, Mizoguchi E, Bhan AK, et al. CD40-deficient mice generated by recombination-activating gene-2-deficient blastocyst complementation. *Proc Natl Acad Sci U S A* 1994;91:12135-9.
25. Jabara H, Laouini D, Tsitsikov E, Mizoguchi E, Bhan A, Castigli E, et al. The binding site for TRAF2 and TRAF3 but not for TRAF6 is essential for CD40-mediated immunoglobulin class switching. *Immunity* 2002;17:265-76.
26. Hasbold J, Lyons AB, Kehry MR, Hodgkin PD. Cell division number regulates IgG1 and IgE switching of B cells following stimulation by CD40 ligand and IL-4. *Eur J Immunol* 1998;28:1040-51.
27. Manis JP, Tian M, Alt FW. Mechanism and control of class-switch recombination. *Trends Immunol* 2002;23:31-9.
28. Igarashi K, Ochiai K, Muto A. Architecture and dynamics of the transcription factor network that regulates B-to-plasma cell differentiation. *J Biochem* 2007;141:783-9.
29. Woodland RT, Fox CJ, Schmidt MR, Hammerman PS, Opferman JT, Korsmeyer SJ, et al. Multiple signaling pathways promote B lymphocyte stimulator dependent B-cell growth and survival. *Blood* 2008;111:750-60.
30. Endo T, Nishio M, Enzler T, Cottam HB, Fukuda T, James DF, et al. BAFF and APRIL support chronic lymphocytic leukemia B-cell survival through activation of the canonical NF-kappaB pathway. *Blood* 2007;109:703-10.
31. Kern C, Cornuel JF, Billard C, Tang R, Rouillard D, Stenou V, et al. Involvement of BAFF and APRIL in the resistance to apoptosis of B-CLL through an autocrine pathway. *Blood* 2004;103:679-88.
32. He B, Chadburn A, Jou E, Schattner EJ, Knowles DM, Cerutti A. Lymphoma B cells evade apoptosis through the TNF family members BAFF/BLyS and APRIL. *J Immunol* 2004;172:3268-79.
33. Chiu A, Xu W, He B, Dillon SR, Gross JA, Sievers E, et al. Hodgkin lymphoma cells express TACI and BCMA receptors and generate survival and proliferation signals in response to BAFF and APRIL. *Blood* 2007;109:729-39.
34. Deenick EK, Hasbold J, Hodgkin PD. Switching to IgG3, IgG2b, and IgA is division linked and independent, revealing a stochastic framework for describing differentiation. *J Immunol* 1999;163:4707-14.
35. Acosta-Rodriguez EV, Craxton A, Hendricks DW, Merino MC, Montes CL, Clark EA, et al. BAFF and LPS cooperate to induce B cells to become susceptible to CD95/Fas-mediated cell death. *Eur J Immunol* 2007;37:990-1000.
36. von Bulow GU, Bram RJ. NF-AT activation induced by a CAML-interacting member of the tumor necrosis factor receptor superfamily. *Science* 1997;278:138-41.
37. Bossen C, Schneider P. BAFF, APRIL and their receptors: Structure, function and signaling. *Semin Immunol* 2006;18:263-75.

METHODS

Q-PCR analysis

Taqman primers with 6-carboxyfluorescein-labeled probes for TACI, Blimp-1, IRF-4, and β_2 -microglobulin were obtained from Applied Biosystems. XBP-1(s) and β_2 -microglobulin genes were amplified by using SYBR

Green based-technology (SuperArray Bioscience) and the following primers (Invitrogen, Carlsbad, Calif): XBP-1(s) forward 5'-GGTCTGCTGAGTCCG CAGCAGG-3', XBP-1(s) reverse 5'-AGGCTTGGTGTATACATGG-3', β_2 -microglobulin-forward 5'-TGACCGGCTTGTA TGGTATC-3', and β_2 -microglobulin-reverse 5'-CAGTGTGAGCCAGGATATAG-3'.

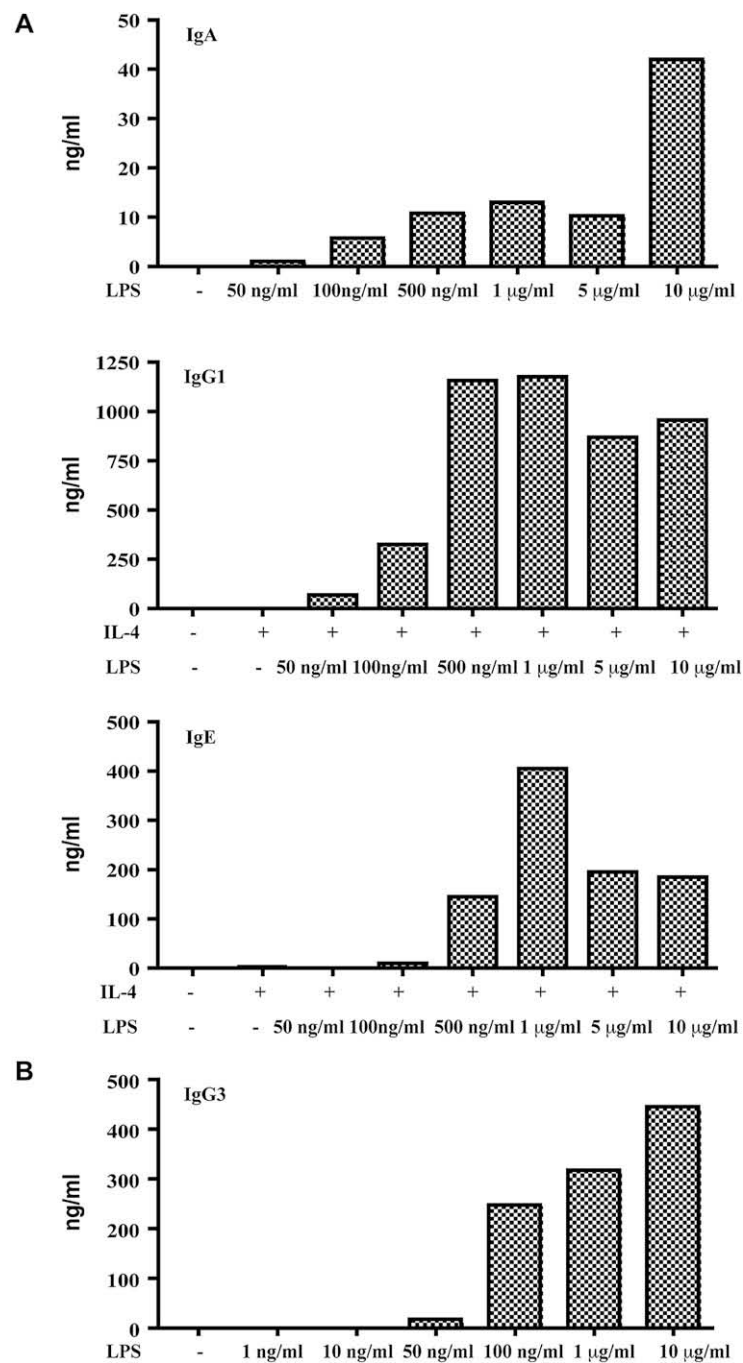


FIG E1. (A) Ig AlgG1, IgE, and (B) IgG3 production by naive B cells in response to different concentrations of LPS. For IgG1 and IgE production, IL-4 was added to the cultures.

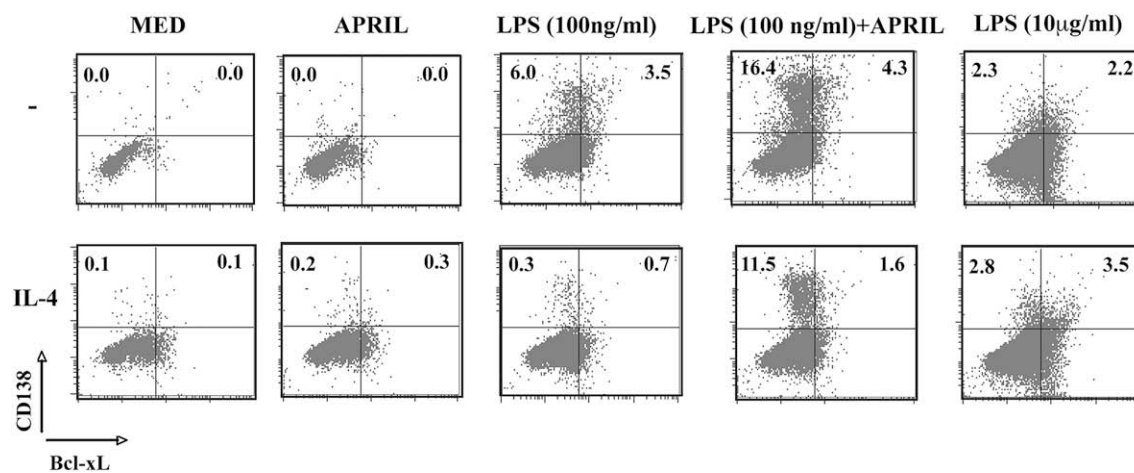


FIG E2. Representative FACS analysis of Bcl-X_L expression in CD138⁺ cells stimulated for 6 days with LPS (100 ng/mL) plus IL-4 in the presence or absence of APRIL or 10 µg/mL LPS plus IL-4. The percentages of positive cells shown in the quadrants are net percentages obtained after subtracting the percentage of cells that were stained by the isotype controls, which in all cases was less than 1.5%. *MED*, Medium.

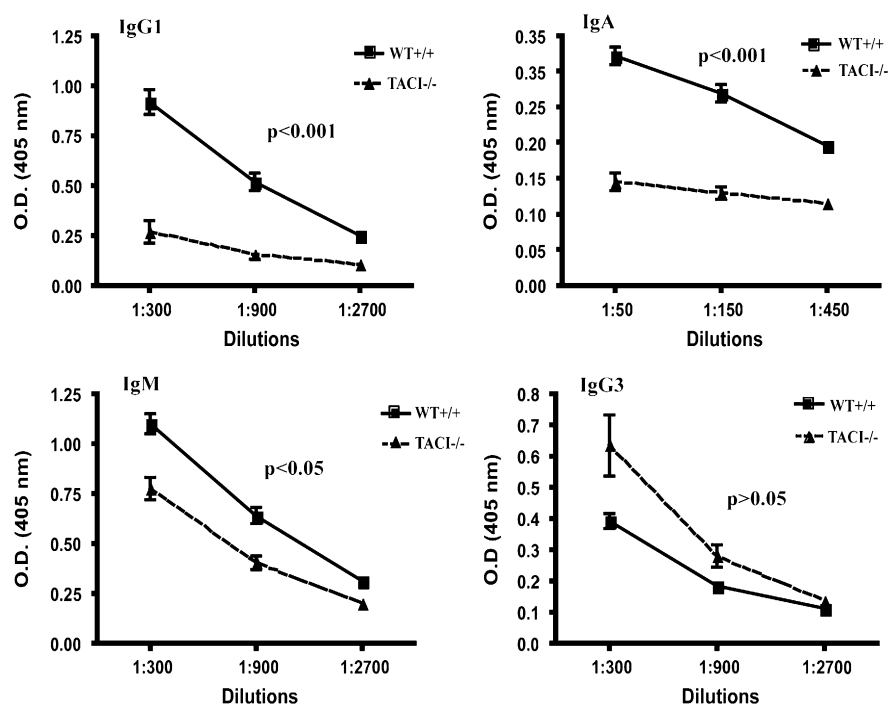


FIG E3. Impaired antibody response to immunization with 10 μ g of TNP-LPS plus 40 μ g of LPS in TACI^{-/-} mice. IgM, IgA, IgG1, and IgG3 anti-TNP antibody levels of TACI^{-/-} and WT mice are shown. Results represent the mean $\pm$ SE of 6 mice. Dilution curves were compared by means of ANOVA.

TABLE E1. APRIL dose response for proliferation and IgA, IgG1, and IgE production of purified splenic naive B cells from BALB/c mice

Concentration of APRIL	Tritiated thymidine incorporation (cpm)	Tritiated thymidine incorporation with IL-4 (cpm)	IgA production (ng/mL)	IgG1 production (ng/mL) with IL-4	IgE production (ng/mL) with IL-4
0	157	463	0	0	0
50 ng/mL	232	1522	0	13.8	8.3
250 ng/mL	364	1526	1.0	18.6	13.5
1 µg/mL	451	1413	1.5	20.3	8.7
4 µg/mL	386	1394	2.5	29.9	5.4

Values represent the mean of triplicate cultures.