

Toll-like receptor 9, transmembrane activator and calcium-modulating cyclophilin ligand interactor, and CD40 synergize in causing B-cell activation

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Background: B cells receive activating signals from T cells through CD40, from microbial DNA through Toll-like receptor (TLR) 9, and from dendritic cells through transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI). TLR9 and CD40 ligation augment TACI-driven B-cell activation, but only the mechanism of synergy between CD40 and TACI has been explored. Synergy between CD40 and TLR9 in B-cell activation is controversial.

Objective: We sought to examine the mechanisms by which TLR9 modulates CD40- and TACI-mediated activation of B cells and to determine whether all 3 receptors synergize to activate B cells.

Methods: Naive murine B cells and human PBMCs were stimulated with combinations of anti-CD40, CpG, and a proliferation inducing ligand in the presence of IL-4. Proliferation was measured by means of tritiated thymidine incorporation. Immunoglobulin production was measured by means of ELISA. Class-switch recombination (CSR) was examined by measuring mRNA for germline transcripts, activation-induced cytidine deaminase (*AICDA*), and mature immunoglobulin transcripts. Plasma cell differentiation was examined by using syndecan-1/CD138 staining and mRNA expression of B lymphocyte-induced maturation protein 1 (*Blimp-1*).

Results: TLR9 synergized with CD40 and TACI in driving CSR and inducing IgG₁ and IgE secretion by naive murine B cells and synergized with TACI in driving B-cell proliferation and plasma cell differentiation. All 3 receptors synergized together in driving murine B-cell proliferation, CSR, plasma cell differentiation, and IgG₁ and IgE secretion. TLR9 synergized with CD40 and TACI in driving IgG secretion in IL-4-stimulated human B cells.

Conclusion: Signals from TLR9, TACI, and CD40 are integrated to promote B-cell activation and differentiation. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: Toll-like receptor 9, CD40, transmembrane activator and calcium-modulating cyclophilin ligand interactor, class-switch recombination, plasma cell, immunoglobulin, B cells

B cells respond to antigens by proliferating and undergoing maturation and differentiation, which include class-switch recombination (CSR), antibody affinity maturation, and generation of memory B cells and plasma cells. These processes are dependent on interactions between ligand-receptor pairs that include CD40 ligand–CD40, B cell–activating factor belonging to the TNF superfamily (BAFF)/a proliferation inducing ligand (APRIL)–transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI), and Toll-like receptor (TLR) ligands–TLRs.^{1–3}

B-cell activation by means of CD40 stimulation is essential for T cell–dependent antibody responses and is the major contributor to immunoglobulin production *in vivo*. This is evidenced by the observation that mice and patients deficient in CD40 ligand (CD40L) or CD40 have severely decreased levels of serum IgG, lack serum IgE, do not mount antigen-specific antibody responses after immunization with T cell–dependent antigens, and lack germinal centers (GCs).^{4–7}

TACI is expressed on a fraction of B cells and binds to the TNF superfamily members BAFF and APRIL.⁸ BAFF is expressed by neutrophils, monocytes, and dendritic cells (DCs) and promotes B-lymphocyte survival and maturation, and APRIL is expressed in monocytes, macrophages, DCs, and activated T cells.⁹ TACI mediates isotype switching by APRIL^{2,10} and synergizes with CD40 and TLR4 to increase immunoglobulin production and generation of CD138⁺ plasma cells *in vitro*.^{11,12} TACI^{−/−} mice have decreased serum IgM, IgG, and IgA levels and deficient antibody responses to the type II T cell–independent antigens pneumococcal polysaccharides and TNP-Ficoll.^{13–16} Furthermore, they have an impaired antibody response to immunization with a suboptimal dose of the type I T cell–independent antigen TNP-LPS, indicating that TACI synergizes with TLR4 *in vivo*.¹² Heterozygous mutations in *TACI* are found in approximately 10% of patients with common variable immunodeficiency (CVID) and in approximately 1% of healthy subjects.^{17,18}

TLRs recognize specific molecular patterns of microbial pathogens and play an essential role in innate immunity to these pathogens. In addition, they play an important role in the adaptive immune responses, in part by promoting the maturation of antigen-presenting DCs to activate T and B cells.^{19,20} The TLR4 ligand LPS and the TLR9 ligand CpG activate isotype switching in B cells to IgG₃ in B cells and to other isotypes in the presence of cytokines.^{21,22} TLRs also recognize endogenous ligands.²³

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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
CD40L: CD40 ligand
CSR: Class-switch recombination
CVID: Common variable immunodeficiency
DC: Dendritic cell
GC: Germinal center
GLT: Germline transcript
sIgG₁: Surface IgG₁
TACI: Transmembrane activator and calcium-modulating cyclophilin ligand interactor
TLR: Toll-like receptor

B cells in secondary lymphoid organs, particularly in GCs, are exposed to activated T cells, which express CD40L, and to DCs, which are a source for BAFF and APRIL. In addition, they might be exposed to exogenous and endogenous TLR ligands released by microbes and dying B cells in GCs. Thus B cells responding to antigen might simultaneously receive signals through CD40, TACI, and TLR9. These 3 receptors might synergize in pairs or altogether to result in optimal B-cell activation and differentiation.

We have previously shown that CD40 upregulates TACI expression and synergizes with TACI in driving plasmablast differentiation and immunoglobulin production in naive B cells.¹¹ TLR9 ligation has been shown to synergize with TACI ligation in driving immunoglobulin production.^{21,24} The mechanisms that underlie this synergy have not been explored. The data on the synergy between TLR9 and CD40 are contradictory.²⁵⁻²⁷ In this work we demonstrate that TLR9 synergizes with CD40 and identify the mechanisms involved in the synergy between TLR9 and CD40 and between TLR9 and TACI. We also demonstrate that signals delivered through CD40, TLR9, and TACI synergize to increase immunoglobulin production by murine naive B cells and identify the mechanisms involved in this synergy. We also show that TLR9 synergizes with CD40 and APRIL in driving IgG production in human PBMCs from healthy subjects.

METHODS**Mice**

BALB/c mice were purchased from Charles River Laboratories (Wilmington, Del). *TACI*^{-/-}, B-cell maturation antigen^{-/-} (*BCMA*^{-/-}), and genetically matched wild-type mice on an Sv129xC57Bl6 background were previously described.^{14,28} All mice were bred and kept in a specific pathogen-free animal facility. All procedures performed on the animals were approved by the Animal Care and Use Committee of the Children's Hospital, Boston, Massachusetts.

Immunoglobulin production and proliferation by murine B cells

Naive B cells were negatively sorted from murine splenocytes by using a cocktail of biotin-conjugated mAbs to CD43, CD11b, Thy1.2, CD138, IgG₁, IgG_{2a/b}, IgG₃, IgA, and IgE and streptavidin magnetic beads (Dynabeads M-280; Invitrogen, Carlsbad, Calif). B cells were cultured at a concentration of 1×10^6 /mL in RPMI containing 10% FCS, L-glutamine, penicillin/streptomycin, and 50 mmol/L 2-mercaptoethanol. Naive B cells were stimulated

with APRIL at 1 μ g/mL (R&D Systems, Minneapolis, Minn); IL-4 at 50 ng/mL (R&D Systems); CpG oligonucleotide 1826 (InvivoGen, San Diego, Calif) at 10 ng/mL, 100 ng/mL, or 3 μ g/mL; anti-CD40 antibody (BD Pharmingen, San Jose, Calif) at 1, 10, or 100 ng/mL; or combinations thereof. After 6 days in culture, supernatants were assayed for IgM, IgA, IgG₃, IgG₁, and IgE by means of ELISA, as previously described.²⁹ After 72 hours, cultures were pulsed with 1 μ Ci of tritiated thymidine for an additional 16 hours and then harvested and scintillation counted.

Surface staining

B cells were stained on day 6 after stimulation with anti-B220–fluorescein isothiocyanate, anti-CD138–phycoerythrin, or anti-IgG1–phycoerythrin (BD Pharmingen) and analyzed by using FACS (BD FACSCalibur), as previously described.¹¹

B-cell survival

B cells were stained on day 3 after stimulation with Annexin V–fluorescein isothiocyanate and propidium iodide (BioVision, Mountain View, Calif) and analyzed by using FACS.

RT-PCR analysis

RNA extracted from 4-day cultures with TRIzol (Invitrogen) was reverse transcribed with Superscript II RT (Invitrogen). PCR primers and conditions used to detect *I ϵ -C ϵ* , *I μ -C ϵ* , *I γ 1-C γ 1*, *I μ -C γ 1*, activation-induced cytidine deaminase (*AICDA*), and β_2 -microglobulin have been previously described.²⁹ Amplified products were separated on agarose gels and stained with ethidium bromide.

Quantitative PCR analysis

Real-time PCR reactions were run on cDNA by using the ABI Prism 7300 (Applied Biosystems, Foster City, Calif). Taqman primers for B lymphocyte-induced maturation protein 1 (*Blimp-1*) were obtained from Applied Biosystems.

Human subjects

Blood was obtained from healthy adult subjects after securing informed consent.

Immunoglobulin production and proliferation by human PBMCs

PBMCs (1.5×10^6 cells/mL) were cultured in complete medium with CpG (0.001 μ mol/L), zzAPRIL (10 nmol/L; ZymoGenetics, Inc, Seattle, Wash), and anti-CD40 mAb 626.1 (10 ng/mL, a kind gift of Dr S. M. Fu, University of Virginia) plus IL-4 (5 ng/mL, R&D Systems) for 14 days.³⁰ Supernatants were harvested, and IgG was assayed by means of ELISA. Net IgG synthesis was calculated by subtracting the amount of IgG in unstimulated cultures.

Statistics

P values were calculated by using the paired *t* test with PRISM software (Prism Software Corp, Irvine, Calif).

RESULTS**CpG synergizes with CD40 ligation in driving immunoglobulin production and proliferation by naive B cells**

A range of concentrations of anti-CD40 (1–100 ng/mL) and CpG (10 ng/mL to 3 μ g/mL) were tested for their ability to drive IgG₁ and IgE production by negatively sorted naive B cells (95%

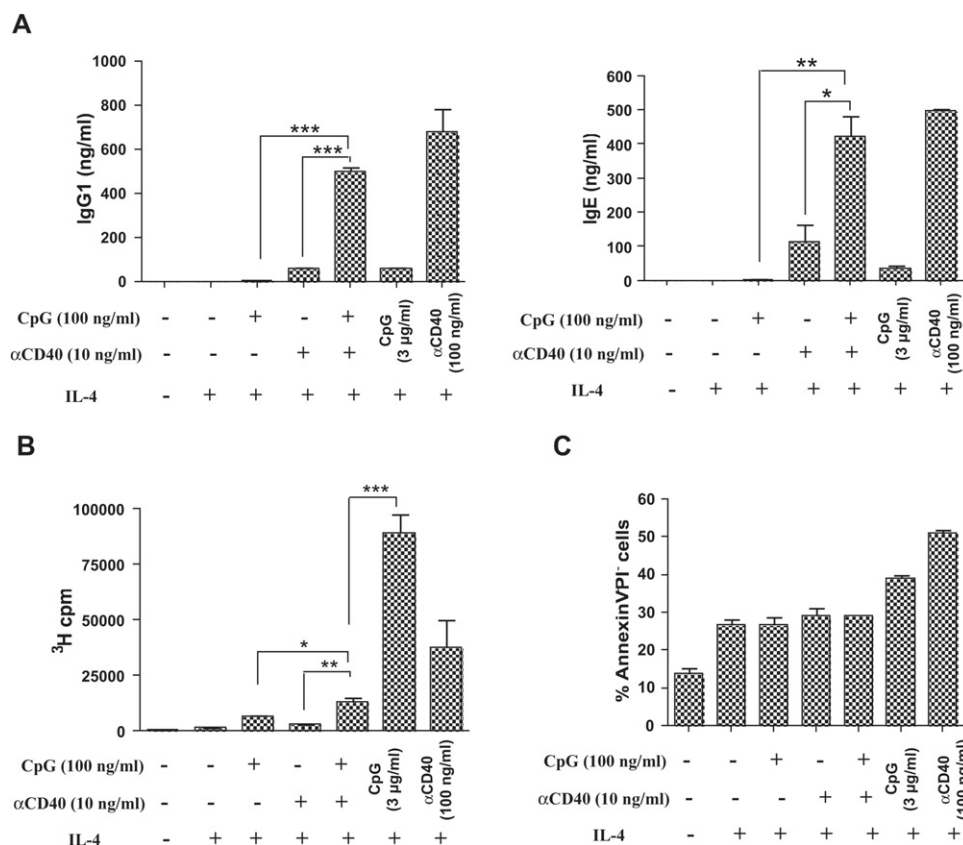


FIG 1. TLR9 synergizes with CD40 in activating naive B cells. **A** and **B**, Synergy between CpG and anti-CD40 in driving IgG₁ and IgE production (Fig 1, A) and proliferation (Fig 1, B) in naive B cells cultured with IL-4. **C**, Effect of addition of CpG to anti-CD40 mAb on B-cell survival. Values represent means $\pm$ SDs of at least 3 experiments. * $P < .05$, ** $P < .01$, *** $P < .001$. Note that in this and subsequent figures, not all bars representing SDs are readily visible.

IgM⁺IgD⁺ cells) from BALB/c mice in the presence of IL-4. On the basis of the results obtained (see Figs E1 and E2 in this article's Online Repository at www.jacionline.org), suboptimal concentrations of 10 ng/mL anti-CD40 and 100 ng/mL CpG were selected to examine for potential synergy between TLR9 and CD40 in driving immunoglobulin production by naive B cells. Fig 1, A, shows that CpG synergized with anti-CD40 in driving IgG₁ and IgE synthesis in the presence of IL-4. The levels of secreted IgG₁ and IgE were comparable with those of B cells stimulated with an optimal concentration of 100 ng/mL anti-CD40 plus IL-4. There was no detectable synergy between CpG and anti-CD40 in driving IgM production (data not shown).

IgG₁ and IgE production by naive B cells involves isotype switching, which is thought to be linked to B-cell proliferation.³¹ Fig 1, B, shows that stimulation with suboptimal concentrations of CpG and anti-CD40 had an additive effect on the proliferation of naive B cells in the presence of IL-4. The level of this proliferation was not significantly different than that induced by an optimal concentration of 100 ng/mL anti-CD40 plus IL-4 but was significantly less than that induced by 3 μg/mL CpG plus IL-4. Staining with Annexin V and propidium iodide revealed no detectable effect of CpG (100 ng/mL) on the survival of naive B cells stimulated with 10 ng/mL anti-CD40 plus IL-4, as determined by the percentage of Annexin V-negative propidium iodide-negative cells (Fig 1, C).

CpG synergizes with CD40 ligation in driving isotype switching

To investigate the potential synergy of TLR9 and CD40 in driving CSR, we examined surface IgG₁ (sIgG₁) expression by naive B cells stimulated for 6 days with suboptimal concentrations of CpG and anti-CD40 in the presence of IL-4. Fig 2, A, shows that suboptimal concentrations of CpG and anti-CD40 synergized in inducing the expression of sIgG₁. The percentage of sIgG₁⁺ cells in these cultures was comparable with those seen in cultures stimulated with optimal concentrations of CpG (3 μg/mL) plus IL-4 or anti-CD40 (100 ng/mL) plus IL-4.

CSR requires expression of germline transcripts (GLTs) and *AICDA* followed by deletional switch recombination and expression of postswitch mature transcripts (I μ -C_H).³² Fig 2, B, shows that suboptimal concentrations of CpG and anti-CD40 synergized in the presence of IL-4 to induce mRNA expression of C ϵ and C γ 1 GLTs, *AICDA*, and I μ -C ϵ and I μ -C γ 1 mature transcripts, as determined by means of RT-PCR. To assess potential synergy between CpG and anti-CD40 on plasma cell differentiation, we examined surface expression of syndecan-1/CD138 and mRNA expression of the plasma cell differentiation factor *Blimp-1*. There was no significant increase in the percentage of CD138⁺ cells or in the level of *Blimp-1* mRNA in cultures stimulated with suboptimal concentrations of CpG and anti-CD40 compared with cultures stimulated with either (Fig 2, C and D). These results suggest that the synergy

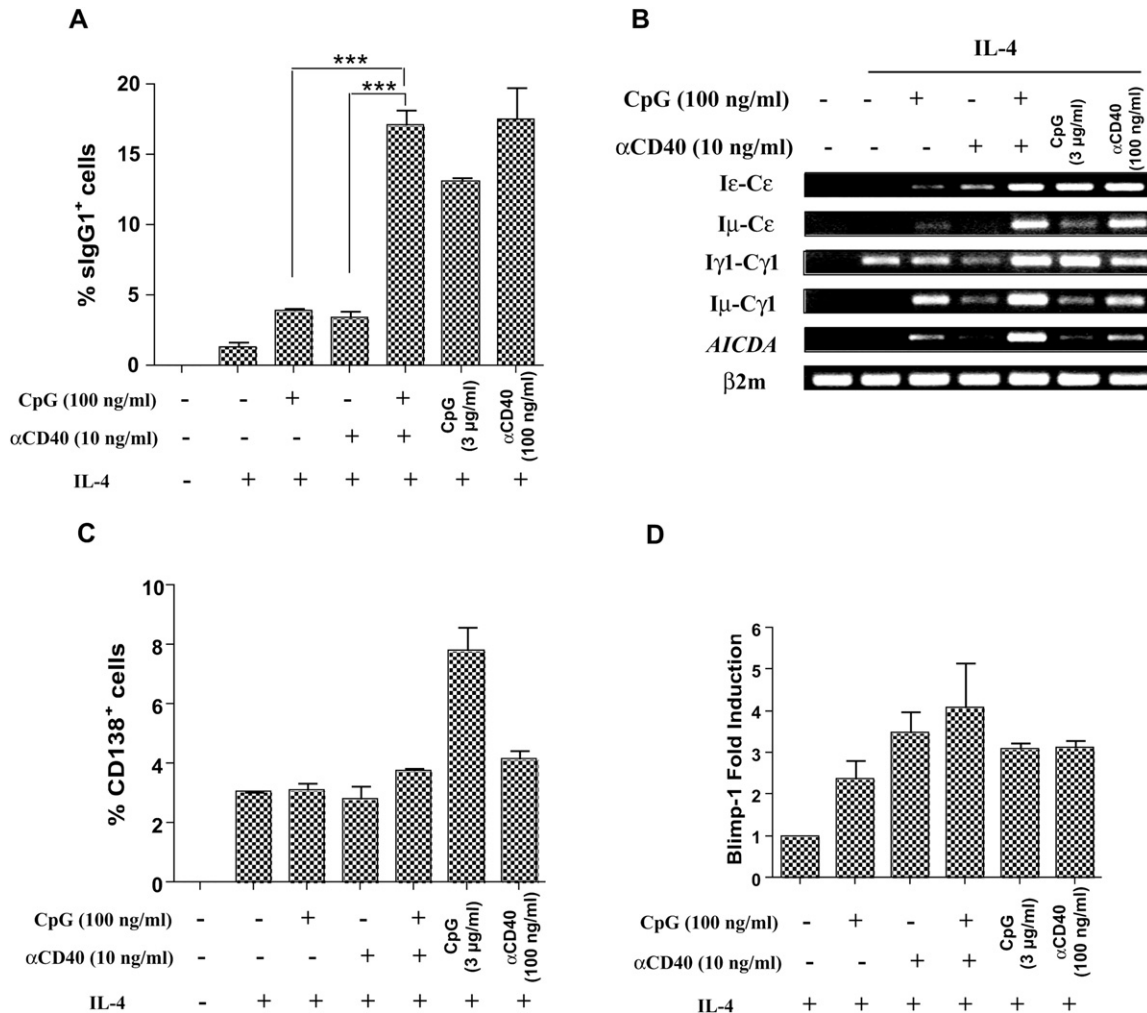


FIG 2. TLR9 synergizes with CD40 in driving isotype switching. **A**, sIgG₁ expression. **B**, Expression of Cε GLT, Iμ-Cε, Cγ1 GLT, Iμ-Cγ1, and *AICDA* mRNA measured by means of RT-PCR. Similar results were obtained in 3 independent experiments. **C**, CD138 surface expression. **D**, *Blimp-1* mRNA expression. Values in Fig 2, A, C, and D, represent means ± SDs of at least 3 experiments. ****P* < .001.

between TLR9 and CD40 is exerted primarily at the level of B-cell proliferation and CSR. It should be noted that there was 10-fold less IgG₁ secretion by B cells stimulated with optimal concentrations of CpG than with optimal concentrations of anti-CD40, despite comparable expression of sIgG₁, *Blimp-1* mRNA, and CD138. Clearly, sIgG₁ expression need not correlate with IgG₁ secretion. There are also data to suggest that neither *Blimp-1* expression nor expression of plasmablast markers is sufficient for a high rate of immunoglobulin secretion.³³

TLR9 synergizes with TACI in driving immunoglobulin production, proliferation, CSR, and plasma cell differentiation by naive B cells

Preincubation of B cells with CpG enhances IgM, IgG, and IgA secretion in response to APRIL.²¹ To examine the mechanism of this enhancement, we first confirmed that suboptimal amounts of CpG (10 ng/mL) synergize with APRIL (1 μg/mL) in driving IgM and IgG₃ by naive B cells (see Fig E3, A, in this article's Online Repository at www.jacionline.org and data not shown). CpG and APRIL also synergized in driving IgG₁ and IgE secretion by naive

B cells in the presence of IL-4 (Fig 3, A) to levels comparable with those observed with optimal amounts of CpG (3 μg/mL) plus IL-4. The 10-fold lower IgG₁ and IgE secretion by B cells stimulated with APRIL plus CpG compared with B cells stimulated with anti-CD40 plus CpG at the same concentration reflects the fact that anti-CD40, even at a concentration of 10 ng/mL, is a more potent B-cell stimulator than APRIL. CpG synergized with APRIL in driving the proliferation of naive B cells in the presence of IL-4 (Fig 3, B) and in its absence (see Fig E3, B). There was no detectable effect of CpG on the survival of naive B cells stimulated with APRIL plus IL-4 (Fig 3, C) or APRIL alone (see Fig E3, C).

CpG synergized with APRIL in inducing sIgG₁ expression by naive B cells (Fig 4, A) and in inducing mRNA expression of Cε GLT, *AICDA*, and Iμ-Cε and Iμ-Cγ1 mature transcripts in these cells (Fig 4, B). Cγ1 GLT expression was not affected. The 2 ligands were additive in inducing the expression of CD138 (Fig 4, C) on naive B cells cultured with IL-4 and in inducing mRNA expression of *Blimp-1* by these cells (Fig 4, D). These results indicate that TLR9 and TACI synergize in causing B-cell proliferation and CSR and have additive effects on plasma cell differentiation.

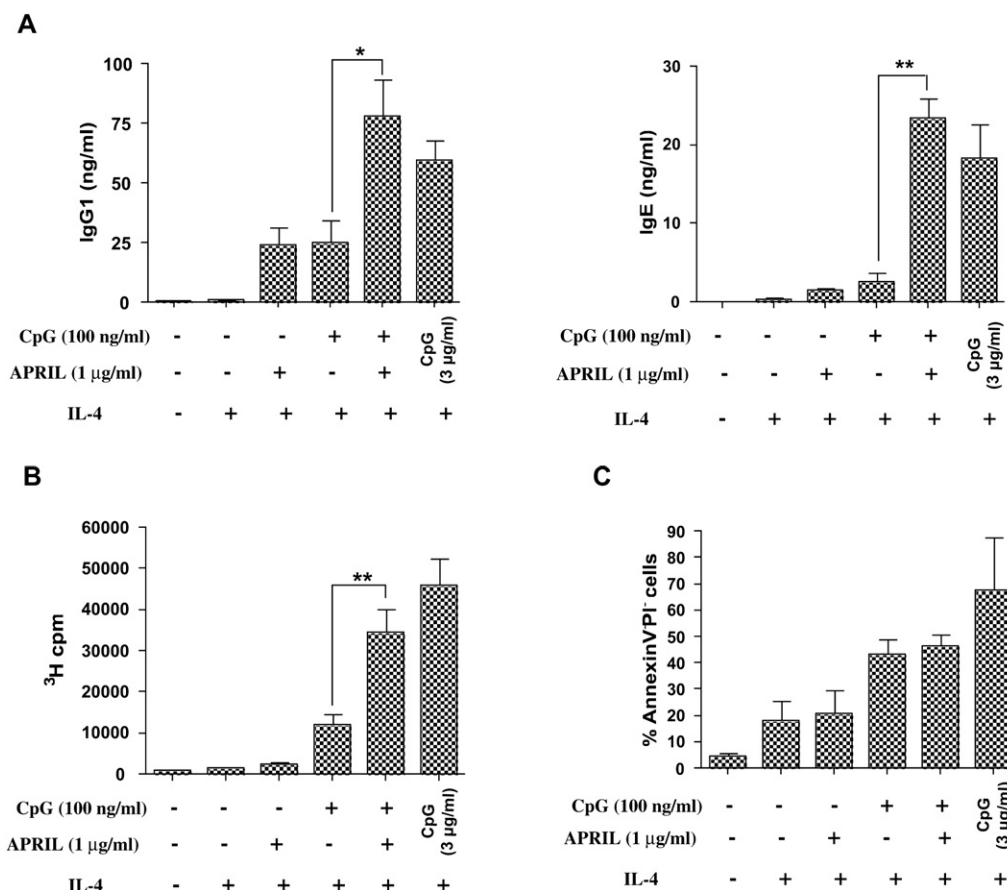


FIG 3. TLR9 synergizes with TAC1 in driving immunoglobulin production and proliferation. **A** and **B**, Synergy between CpG and APRIL in driving IgG₁ and IgE production (Fig 3, A) and proliferation (Fig 3, B) in naive B cells stimulated with IL-4. **C**, Effect of addition of CpG to APRIL on B-cell survival. Values represent means $\pm$ SDs of at least 3 experiments. * $P < .05$, ** $P < .01$.

APRIL binds to TAC1, BCMA, and heparan sulfate proteoglycans on B cells.^{8,34} Synergy between CpG and APRIL in driving IgG₁ and IgE production in B cells stimulated with IL-4 was strictly dependent on TAC1 because it was preserved in B cells from BCMA^{-/-} mice but not in B cells from TAC1^{-/-} mice (see Fig E4 in this article's Online Repository at www.jacionline.org).

Triple synergy between APRIL, CpG, and anti-CD40 in driving B-cell activation and differentiation

We next tested for potential triple synergy among TLR9, CD40, and TAC1 in B-cell activation. Suboptimal concentrations of CpG and anti-CD40 synergized with APRIL in driving IgG₁ and IgE secretion and proliferation of naive B cells cultured with IL-4 (Fig 5, A and B). The 10-fold lower IgG₁ and IgE levels secreted by B cells stimulated with all 3 ligands compared with B cells stimulated with anti-CD40 plus CpG is likely due to the fact that we used a 10-fold lower concentration of anti-CD40 in the triple-synergy than in the double-synergy experiment (1 vs 10 ng/mL). There was no detectable synergy among the 3 ligands on B-cell survival in these cultures (data not shown). CpG, anti-CD40, and APRIL also synergized in inducing the expression of sIgG₁ and of mRNA for C ϵ GLT, *AICDA*, and I μ -C ϵ and I μ -C γ 1 mature transcripts (Fig 5, C and D). C γ 1 GLT expression

was not affected. The 3 ligands were additive in inducing the expression of CD138 on naive B cells cultured with IL-4 (Fig 5, E) and synergized in inducing mRNA expression of *Blimp-1* by these cells (Fig 5, F).

TLR9 synergizes with CD40 and APRIL in driving IgG synthesis by human B cells

We examined whether the synergy observed in B cells from genetically homogeneous mice would apply to B cells from outbred human subjects. On the basis of preliminary dose-response experiments, we stimulated PBMCs from healthy adult subjects with suboptimal concentrations of CpG at 0.001 µmol/L, zzAPRIL at 10 nmol/L, and anti-CD40 mAb at 10 ng/mL. All cultures received IL-4 (5 ng/mL). CpG synergized both with anti-CD40 and zzAPRIL in driving IgG secretion by human PBMCs (Fig 6).

DISCUSSION

The present work demonstrates that TLR9, CD40, and TAC1 synergize, when used at suboptimal concentrations, to drive B cells to secrete immunoglobulins.

Our data clearly demonstrate that suboptimal concentrations of CpG and anti-CD40 synergize in driving IgG₁ and IgE secretion

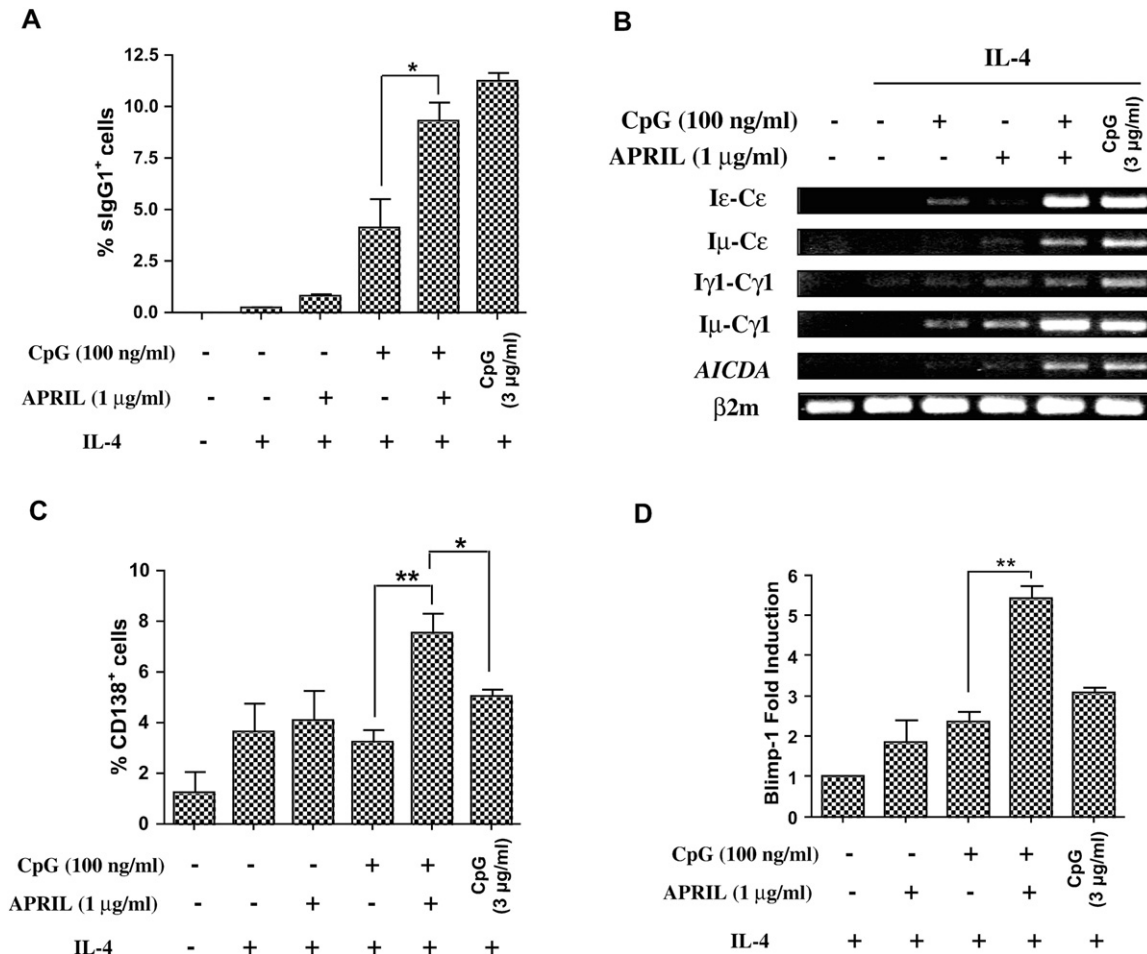


FIG 4. TLR9 and TAC1 synergize in driving isotype switching and have an additive effect on plasma cell differentiation. **A**, sIgG₁ expression. **B**, Expression of Cε GLT, Iµ-Cε, Cγ1 GLT, Iµ-Cγ1, and *AICDA* mRNA. Similar results were obtained in 3 independent experiments. **C**, CD138 surface expression. **D**, *Blimp-1* mRNA expression. Values in Fig 4, A, C, and D, represent means ± SDs of at least 3 experiments. **P* < .05, ***P* < .01.

in naive murine B cells cultured in the presence of IL-4 (Fig 1, A). Simultaneous ligation of TLR9 and CD40 had additive effects on B-cell proliferation (Fig 1, B) and synergized in driving CSR, as evidenced by synergistic induction of sIgG₁ expression, Cε and Cγ1 germline transcription, *AICDA* expression, and expression of mature IgG₁ and IgE transcripts (Fig 2, A and B). There was no detectable synergy exerted on plasma cell differentiation, as measured by expression of surface CD138 and *Blimp-1* mRNA (Fig 2, C and D). Thus CSR is a major target for the synergy between CD40 and TLR9. TLR9 upregulates CD40 expression on human and murine B cells.³⁵⁻³⁷ However, in contrast to what has been reported in murine macrophages,³⁸ CD40 ligation did not upregulate *TLR9* mRNA expression in murine B cells (data not shown). Enhanced signaling caused by increased CD40 receptor expression might contribute to the synergy between CD40 and TLR9 in driving CSR immunoglobulin production.

Previous studies, which examined synergy between TLR9 and CD40 in B-cell activation, have reached contradictory conclusions. TLR9 and CD40 have been reported to synergize in driving IgG synthesis, proliferation, and expression of the activation markers CD86, CD25, and CD23 in human B cells.²⁶ However, CpG was reported to inhibit CD40/IL-4-driven IgE synthesis in

human B cells and IgG₁ and IgE synthesis in murine B cells.²⁵⁻²⁷

The discrepancy between these results and ours might be related to the use of different concentrations of ligands. Previous murine studies used concentrations of CpG (3 µmol/L, equivalent to 20 µg/mL) and CD40 (3 and 10 µg/mL) that cause strong B-cell activation.^{25,27} We have confirmed the inhibitory effect of CpG on CD40 induction of IgE and IgG₁ synthesis at these concentrations (data not shown). Signaling pathways activated by different concentrations might differ, resulting in opposite effects on B-cell activation. The previous studies on human B cells have used CD40L-transfected CHO cells,²⁶ making it difficult to determine the effective concentration of CD40L. In the present work we used suboptimal ligand concentrations for both TLR9 and CD40. The concentrations of CD40 and TLR9 ligands to which B cells are exposed in lymphoid tissues *in vivo* are not known. Our data suggest that synergy would occur when these concentrations are suboptimal.

TLR9 synergized with TAC1 in driving IgG₁ and IgE secretion proliferation (Fig 3, A and B) and CSR (Fig 4, A and B) in IL-4-stimulated naive B cells and exerted additive effects on plasma cell differentiation, as measured by the expression of surface CD138 and *Blimp-1* mRNA levels (Fig 4, C and D). Thus

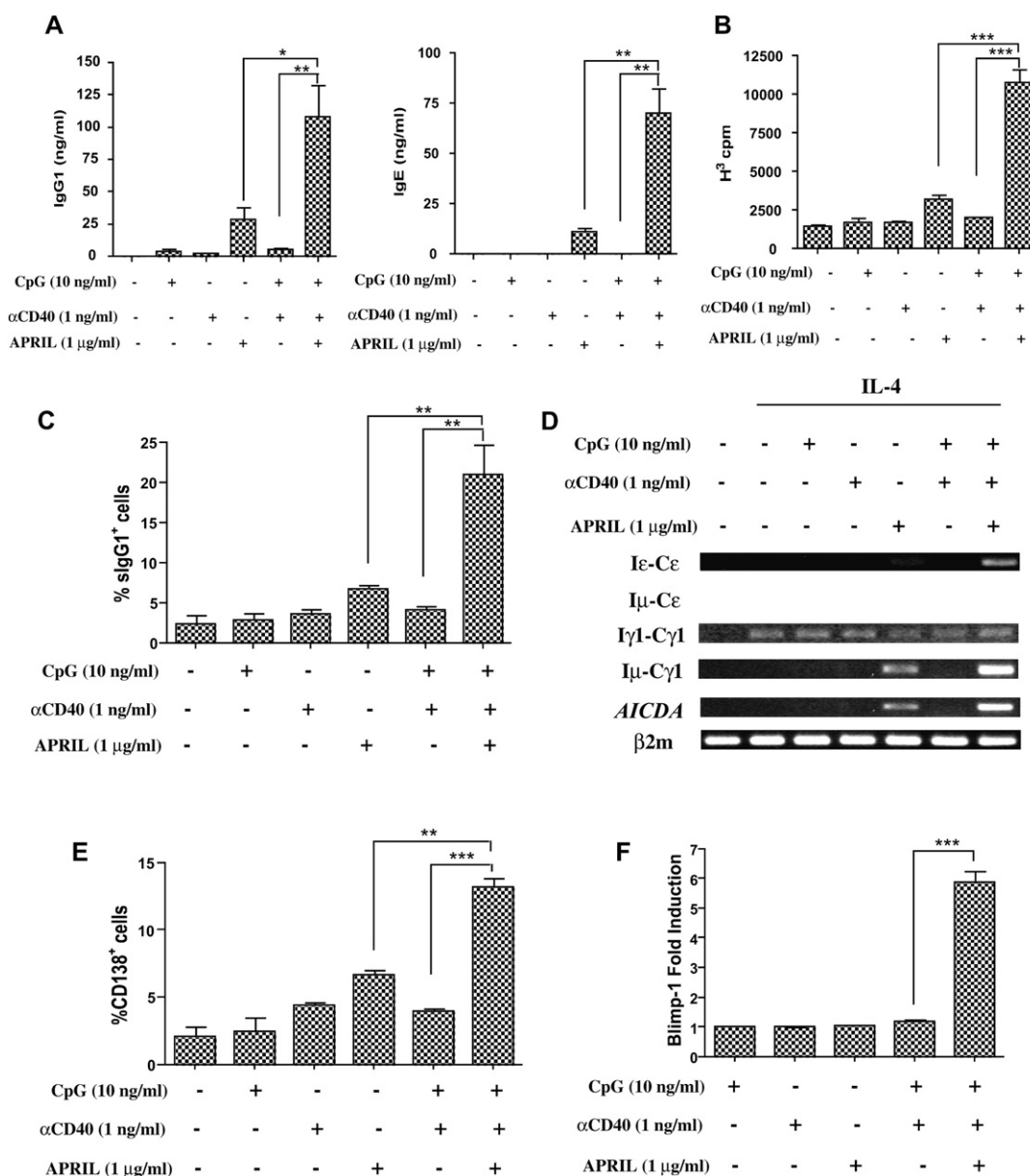


FIG 5. Triple synergy between TLR9, CD40, and TACI in driving B-cell activation and differentiation in the presence of IL-4. IgG₁ and IgE production (**A**) and proliferation (**B**), sIgG₁ expression (**C**), molecular events involved in CSR (**D**), CD138 expression (**E**), and *Blimp-1* mRNA expression (**F**) by naive B cells cultured with IL-4 are shown. Values in Fig 5, A, B, C, E, and F, represent means $\pm$ SDs of at least 3 experiments. Fig 5, D, is representative 3 independent experiments. * P < .05, ** P < .01, *** P < .001.

TLR9 and TACI cooperate at multiple steps to promote immunoglobulin production, which include clonal expansion, CSR, and plasma cell differentiation. TLR9 ligation upregulates TACI expression on murine B cells.^{21,39} However, APRIL stimulation does not upregulate *TLR9* mRNA expression (data not shown). Enhanced TACI signaling because of increased TACI receptor expression might contribute to the synergy between TLR9 and TACI.

We demonstrated that CD40, TLR9, and TACI synergize together to drive B-cell activation. Suboptimal concentrations of CpG and anti-CD40 synergized with APRIL to cause B-cell proliferation, CSR, and IgG₁ and IgE production and exerted additive effects on CD138 and *Blimp-1* expression (Fig 5). These

results suggest that B cells exposed *in vivo* to suboptimal concentrations of ligands for CD40, TLR9, and TACI integrate signals from all 3 receptors to achieve optimal activation and antibody production. These might include nuclear factor κ B activated by all 3 receptors,^{19,40,41} nuclear factor of activated T cells activated by TACI,⁴¹ and signal transducer and activator of transcription 3 activated by TLR9.⁴² Nuclear factor κ B is critical for *AICDA* induction,⁴³ and signal transducer and activator of transcription 3 is critical for *Blimp-1* expression and plasma cell differentiation.^{44,45}

Activated T cells and follicular DCs are well-established sources of CD40 and TACI ligands, respectively, in GCs.^{46,47} Mitochondrial DNA released by damaged tissue has been shown to

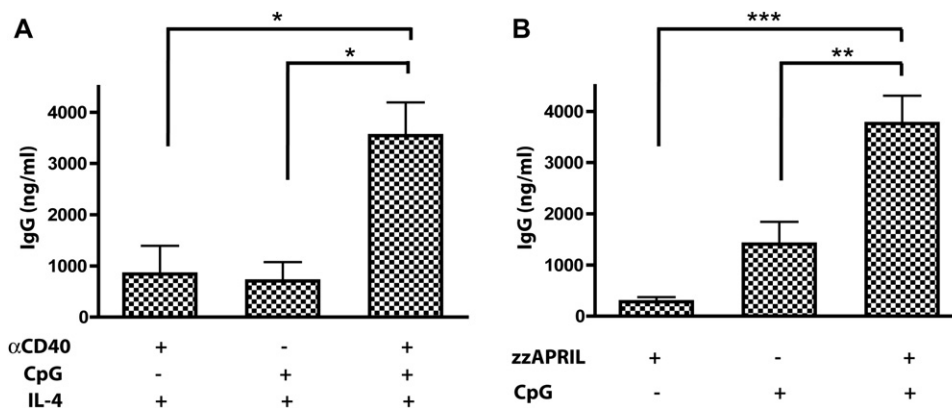


FIG 6. Synergy between CpG, anti-CD40, and APRIL in PBMCs from healthy subjects. Synergy between CpG and anti-CD40 (**A**, $n = 3$) and between CpG and APRIL (**B**, $n = 13$) in driving IgG production by IL-4-stimulated PBMCs.

engage TLR9 in neutrophils,⁴⁸ raising the possibility that mitochondrial DNA released by dying B cells might deliver a TLR9 signal to neighboring B cells that have been selected in GCs. Although we have used naive B cells to study the cellular and molecular events that are involved in B-cell differentiation from naive cells to plasmablasts, synergy between CD40 TACI and TLR9 is likely to operate not only in naive B cells but also in B cells after they have undergone CSR.

Our current study on the synergy between TLR9, CD40, and TACI is unique in that it has used purified naive B cells and addressed the molecular mechanisms of synergy between these receptors. In addition, it demonstrates for the first time that B cells can integrate signals from all 3 receptors to achieve optimal activation. Furthermore, it demonstrates that signals from TLR9, CD40, and TACI also synergize to drive immunoglobulin production by human B cells. Patients with CVID have an impaired response to TLR9.^{49,50} A subset of these patients have mutations in TACI with impaired response to APRIL.^{17,18} Another subset exhibits defective CD40L expression.^{51,52} Synergy between TLR9, CD40, and TACI might be disrupted in these patients, and defects in TLR9, CD40, and TACI signaling might cooperate to impair B-cell immunoglobulin production in patients with CVID.

Clinical implications: Stimulation of B cells through CD40, TLR9, and TACI results in their optimal activation. Defects in these pathways might cooperate to impair B-cell immunoglobulin production in patients with CVID.

REFERENCES

- Fuleihan R, Ramesh N, Geha RS. Role of CD40-CD40L interaction in Ig isotype switching. *Curr Opin Immunol* 1993;5:963-7.
- 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.
- He B, Qiao X, Cerutti A. CpG DNA induces IgG class switch DNA recombination by activating human B cells through an innate pathway that requires TLR9 and cooperates with IL-10. *J Immunol* 2004;173:4479-91.
- Fuleihan R, Ramesh N, Loh R, Jabara H, Rosen RS, Chatila T, et al. Defective expression of the CD40 ligand in X chromosome-linked immunoglobulin deficiency with normal or elevated IgM. *Proc Natl Acad Sci U S A* 1993;90:2170-3.
- Ferrari S, Giliani S, Insalaco A, Al-Ghonaim A, Soresina AR, Loubser M, et al. Mutations of CD40 gene cause an autosomal recessive form of immunodeficiency with hyper IgM. *Proc Natl Acad Sci U S A* 2001;98:12614-9.
- 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.
- Xu J, Foy TM, Laman JD, Elliott EA, Dunn JJ, Waldschmidt TJ, et al. Mice deficient for the CD40 ligand. *Immunity* 1994;1:423-31.
- Mackay F, Kalled SL. TNF ligands and receptors in autoimmunity: an update. *Curr Opin Immunol* 2002;14:783-90.
- Mackay F, Ambrose C. The TNF family members BAFF and APRIL: the growing complexity. *Cytokine Growth Factor Rev* 2003;14:311-24.
- 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.
- 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.
- Ozcan E, Garibyan L, Lee JJ, Bram RJ, Lam KP, Geha RS. Transmembrane activator, calcium modulator, and cyclophilin ligand interactor drives plasma cell differentiation in LPS-activated B cells. *J Allergy Clin Immunol* 2009;123:1277-86, e5.
- 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.
- Lee JJ, Rauter I, Garibyan L, Ozcan E, Sannikova T, Dillon SR, et al. The murine equivalent of the A181E TACI mutation associated with common variable immunodeficiency severely impairs B-cell function. *Blood* 2009;114:2254-62.
- Castigli E, Wilson SA, Garibyan L, Rachid R, Bonilla F, Schneider L, et al. TACI is mutant in common variable immunodeficiency and IgA deficiency. *Nat Genet* 2005;37:829-34.
- Salzer U, Chapel HM, Webster AD, Pan-Hammarstrom Q, Schmitt-Graeff A, Schlesier M, et al. Mutations in TNFRSF13B encoding TACI are associated with common variable immunodeficiency in humans. *Nat Genet* 2005;37:820-8.
- 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, Sporri R. 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.
- 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.
- Bauer S, Muller T, Hamm S. Pattern recognition by Toll-like receptors. *Adv Exp Med Biol* 2009;653:15-34.
- Kanswal S, Katsenelson N, Selvapandian A, Bram RJ, Akkoyunlu M. Deficient TACI expression on B lymphocytes of newborn mice leads to defective Ig secretion in response to BAFF or APRIL. *J Immunol* 2008;181:976-90.
- Liu M, Ohnishi N, Ni L, Akira S, Bacon KB. CpG directly induces T-bet expression and inhibits IgG1 and IgE switching in B cells. *Nat Immunol* 2003;4:687-93.

26. Gantner F, Hermann P, Nakashima K, Matsukawa S, Sakai K, Bacon KB. CD40-dependent and -independent activation of human tonsil B cells by CpG oligodeoxynucleotides. *Eur J Immunol* 2003;33:1576-85.
27. Peng SL, Li J, Lin L, Gerth A. The role of T-bet in B cells. *Nat Immunol* 2003;4:1041.
28. 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.
29. 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.
30. Brodeur SR, Angelini F, Bacharier LB, Blom AM, Mizoguchi E, Fujiwara H, et al. C4b-binding protein (C4BP) activates B cells through the CD40 receptor. *Immunity* 2003;18:837-48.
31. 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.
32. Manis JP, Tian M, Alt FW. Mechanism and control of class-switch recombination. *Trends Immunol* 2002;23:31-9.
33. Taubenheim N, von Hornung M, Durandy A, Warnatz K, Corcoran L, Peter HH, et al. Defined blocks in terminal plasma cell differentiation of common variable immunodeficiency patients. *J Immunol* 2005;175:5498-503.
34. 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.
35. Jiang W, Lederman MM, Harding CV, Rodriguez B, Mohner RJ, Sieg SF. TLR9 stimulation drives naive B cells to proliferate and to attain enhanced antigen presenting function. *Eur J Immunol* 2007;37:2205-13.
36. Carpenter EL, Mick R, Ruter J, Vonderheide RH. Activation of human B cells by the agonist CD40 antibody CP-870,893 and augmentation with simultaneous toll-like receptor 9 stimulation. *J Transl Med* 2009;7:93.
37. Brummel R, Lenert P. Activation of marginal zone B cells from lupus mice with type A(D) CpG-oligodeoxynucleotides. *J Immunol* 2005;174:2429-34.
38. Buhtoiarov IN, Lum HD, Berke G, Sondel PM, Rakhmilevich AL. Synergistic activation of macrophages via CD40 and TLR9 results in T cell independent antitumor effects. *J Immunol* 2006;176:309-18.
39. 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.
40. van Kooten C, Banchereau J. Functions of CD40 on B cells, dendritic cells and other cells. *Curr Opin Immunol* 1997;9:330-7.
41. 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.
42. Kortylewski M, Kujawski M, Herrmann A, Yang C, Wang L, Liu Y, et al. Toll-like receptor 9 activation of signal transducer and activator of transcription 3 constrains its agonist-based immunotherapy. *Cancer Res* 2009;69:2497-505.
43. Dedeoglu F, Horwitz B, Chaudhuri J, Alt FW, Geha RS. Induction of activation-induced cytidine deaminase gene expression by IL-4 and CD40 ligation is dependent on STAT6 and NFkappaB. *Int Immunol* 2004;16:395-404.
44. Diehl SA, Schmidlin H, Nagasawa M, van Haren SD, Kwakkenbos MJ, Yasuda E, et al. STAT3-mediated up-regulation of BLIMP1 Is coordinated with BCL6 down-regulation to control human plasma cell differentiation. *J Immunol* 2008;180:4805-15.
45. Fuhler GM, Baanstra M, Chesik D, Somasundaram R, Seckinger A, Hose D, et al. Bone marrow stromal cell interaction reduces syndecan-1 expression and induces kinomic changes in myeloma cells. *Exp Cell Res* 2010;316:1816-28.
46. Vinuesa CG, Linterman MA, Goodnow CC, Randall KL. T cells and follicular dendritic cells in germinal center B-cell formation and selection. *Immunol Rev* 2010;237:72-89.
47. Badr G, Borhis G, Lefevre EA, Chaoul N, Deshayes F, Dessirier V, et al. BAFF enhances chemotaxis of primary human B cells: a particular synergy between BAFF and CXCL13 on memory B cells. *Blood* 2008;111:2744-54.
48. Zhang Q, Raoof M, Chen Y, Sumi Y, Sursal T, Junger W, et al. Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature* 2010;464:104-7.
49. Cunningham-Rundles C, Radigan L, Knight AK, Zhang L, Bauer L, Nakazawa A. TLR9 activation is defective in common variable immune deficiency. *J Immunol* 2006;176:1978-87.
50. Yu JE, Knight AK, Radigan L, Marron TU, Zhang L, Sanchez-Ramon S, et al. Toll-like receptor 7 and 9 defects in common variable immunodeficiency. *J Allergy Clin Immunol* 2009;124:349-56, e1-3.
51. Farrington M, Grosmaire LS, Nonoyama S, Fischer SH, Hollenbaugh D, Ledbetter JA, et al. CD40 ligand expression is defective in a subset of patients with common variable immunodeficiency. *Proc Natl Acad Sci U S A* 1994;91:1099-103.
52. Brugnani D, Airo P, Lebovitz M, Malacarne F, Ugazio AG, Notarangelo LD, et al. CD4+ cells from patients with Common Variable Immunodeficiency have a reduced ability of CD40 ligand membrane expression after in vitro stimulation. *Pediatr Allergy Immunol* 1996;7:176-9.

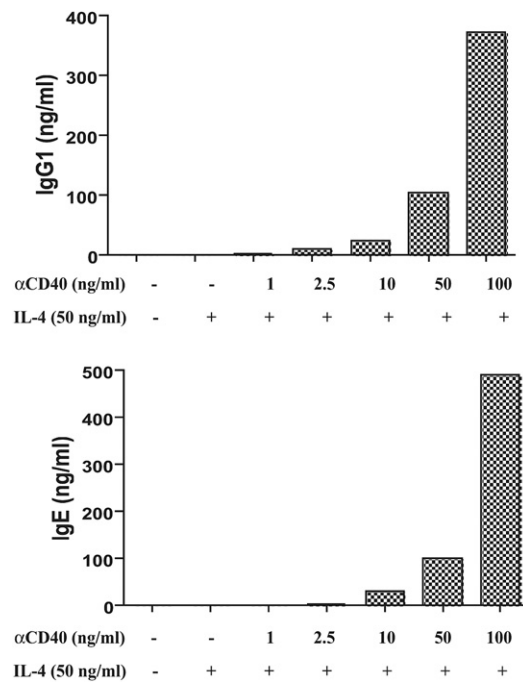


FIG E1. IgG₁ and IgE production by naive B cells in response to stimulation with different concentrations of anti-CD40 mAb plus IL-4. The experiment shown is representative of 3.

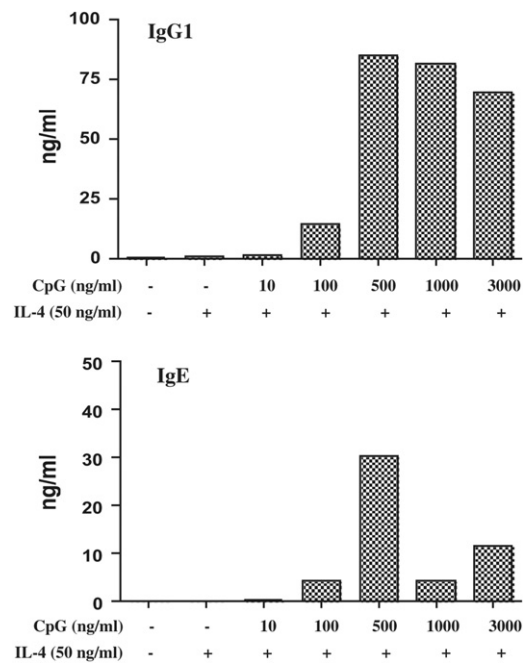


FIG E2. IgG₁ and IgE production by naive B cells in response to stimulation with different concentrations of CpG plus IL-4. The experiment shown is representative of 3.

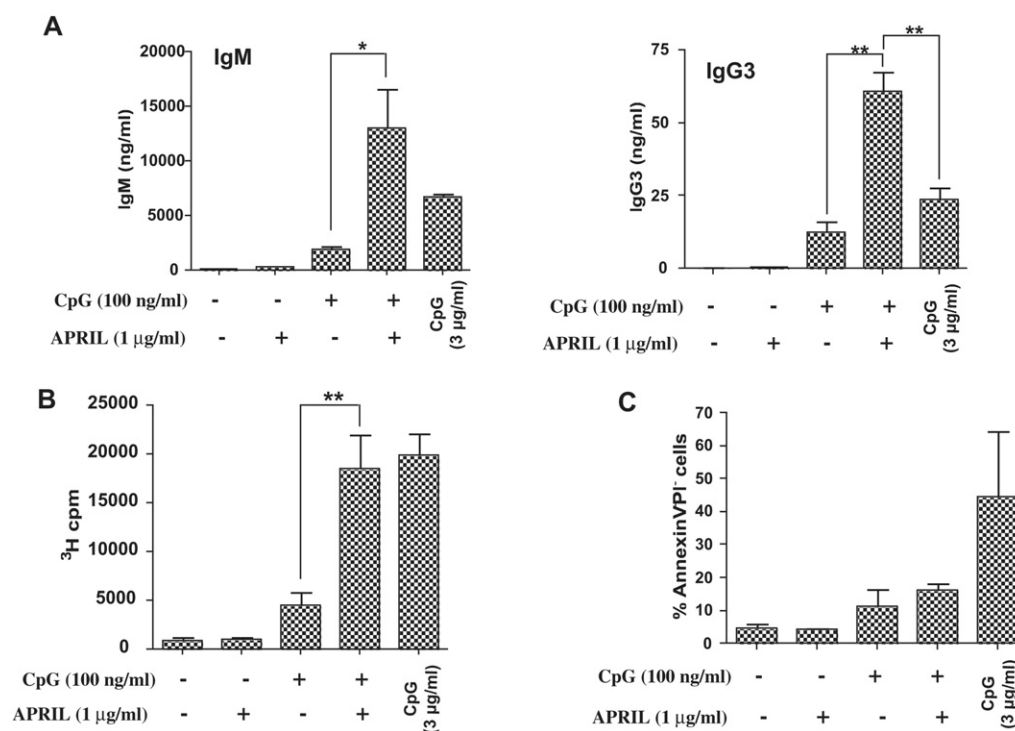


FIG E3. CpG synergizes with TACI ligation in driving IgM and IgG₃ production and proliferation. **A** and **B**, Synergy between CpG and APRIL in driving IgM and IgG₃ production (Fig E3, A) and proliferation (Fig E3, B) in naive B cells. **C**, Effect of addition of CpG to APRIL on B-cell survival. Values represent means $\pm$ SDs of at least 3 experiments. * $P < .05$, ** $P < .01$. At the scale used, not all bars representing SDs are readily visible.

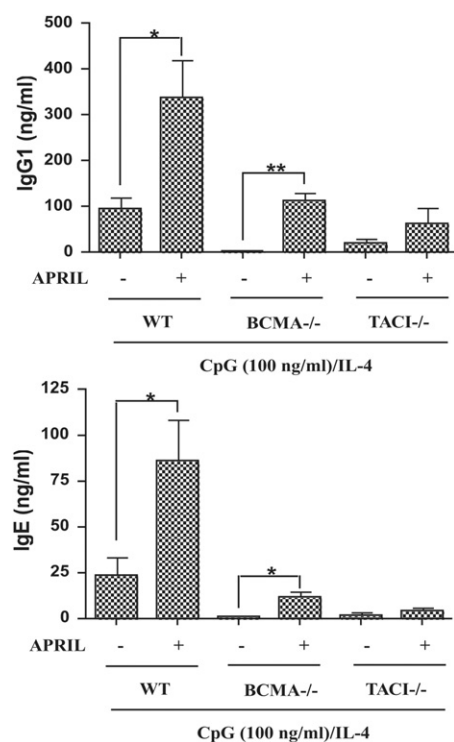


FIG E4. APRIL enhancement of CpG-driven immunoglobulin production is dependent on TACI. Effect of APRIL (1 μ g/mL) on IgG₁ and IgE production by naive B cells from TACI^{-/-} mice, BCMA^{-/-} mice, and genetically matched wild-type control animals. Values represent means $\pm$ SDs of at least 3 experiments. * P < .05, ** P < .01. At the scale used, not all bars representing SDs are readily visible. Variations in absolute amounts of immunoglobulins secreted by B cells from the 3 groups of mice studied likely reflect their mixed Sv129xC57Bl6 background.