

Transmembrane activator and calcium modulator and cyclophilin ligand interactor enhances CD40-driven plasma cell differentiation

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Background: Transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) is a receptor used by B cell-activating factor of the TNF family and a proliferation-inducing ligand (APRIL) to induce isotype switching independently of CD40 and is mutated in patients with common variable immunodeficiency.

Objective: We sought to determine whether TACI and CD40 cooperate in inducing class switch recombination and immunoglobulin production.

Methods: Naive mouse B cells were stimulated with suboptimal concentrations of anti-CD40 plus IL-4 in the presence or absence of APRIL or anti-TACI. IgG₁ and IgE production was measured by means of ELISA. mRNA for C γ ₁ and C ϵ germ-line transcripts, activation-induced cytidine deaminase, and mature γ ₁ and ϵ transcripts were measured by means of RT-PCR. Plasmablasts were enumerated by using syndecan-1/CD138 staining. Interferon regulatory factor 4, B lymphocyte-induced maturation protein 1, and *IL6* mRNA expression was measured by using quantitative PCR.

Results: TACI ligation enhanced IgG₁ and IgE secretion by naive murine B cells stimulated by anti-CD40 plus IL-4, with little effect on B-cell proliferation or class switch recombination. In contrast, TACI ligation of anti-CD40 plus IL-4-stimulated B cells induced a significant increase in syndecan-1/CD138-positive cells. TACI ligation caused a modest but significant increase in the expression of interferon regulatory factor 4, with no detectable change in B lymphocyte-induced maturation protein 1 expression.

Conclusion: TACI and CD40 signaling converge to promote B-cell differentiation into plasmablasts.

Clinical implications: Our data suggest that TACI dysfunction could contribute to the impaired antibody response to

T-dependent antigens in common variable immunodeficiency. (*J Allergy Clin Immunol* 2007;120:885-91.)

Key words: CD40, transmembrane activator and calcium modulator and cyclophilin ligand interactor, common variable immunodeficiency, plasma cell, immunoglobulin, B cells

Humoral immune response to antigens is characterized by the production of high-affinity antibodies by B lymphocytes, which terminally differentiate into plasma cells. B cells encounter antigens in the secondary lymphoid organs, where they proliferate and undergo maturation and differentiation that includes class switch recombination (CSR), affinity maturation, and differentiation into memory B cells or antibody-secreting plasma cells. These processes are initiated and regulated by signals derived from specific ligand-receptor interactions, such as between CD40 ligand (CD40L, CD154) and its receptor, CD40; between a proliferation-inducing ligand (APRIL; CD256, TNFSF13) and B cell-activating factor of the TNF family (CD257, TNFSF13B) and their receptor, transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI; CD267, TNFSF13B)^{1,2}; and between Toll-like receptor (TLR) ligands and their receptors expressed on B cells.³

Differentiation of activated B cells into plasma cells involves repression of genes typically expressed by germinal center (GC) B cells, such as *Pax5*, activation-induced cytidine deaminase (*AICDA*), and *Bcl-6*, and up-regulation of plasma cell-specific genes that include the transcription factors *Prdm1/B* lymphocyte-induced maturation protein 1 (Blimp1), interferon regulatory factor 4 (*IRF4*), and X-box binding protein 1 and the proteoglycan syndecan-1/CD138. Both Blimp1 and IRF4 regulate the expression of X-box binding protein 1,⁴ and B cells deficient in Blimp1 or IRF4 are impaired in their ability to undergo plasma cell differentiation.^{5,6} It has been previously shown that Blimp1 expression is strongly induced by TLR4 ligation but weakly by CD40 ligation in murine B cells.⁷ CD40 ligation induces expression of *IRF4* in human B-cell lines,⁸ but no data have been published on primary mouse B cells.

We and others^{9,10} recently found that the *TNFSF13B* gene, which encodes for TACI, is mutated in approximately

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Abbreviations used

AICDA:	Activation-induced cytidine deaminase
APRIL:	A proliferation-inducing ligand
BCMA:	B-cell maturation antigen
Blimp1:	B lymphocyte-induced maturation protein 1
CFSE:	Carboxyfluorescein succinimidyl ester
CSR:	Class switch recombination
CVID:	Common variable immunodeficiency
GC:	Germinal center
GLT:	Germ-line transcript
HSPG:	Heparan sulfate proteoglycan
IRF4:	Interferon regulatory factor 4
PG:	Prostaglandin
TACI:	Transmembrane activator and calcium modulator and cyclophilin ligand interactor
TLR:	Toll-like receptor
WT:	Wild-type

10% of patients with common variable immunodeficiency (CVID),¹¹ a disorder characterized by recurrent infections, hypogammaglobulinemia, defective antibody production, and failure of B cells to differentiate into memory cells and plasma cells.¹² Naive B cells from patients with CVID with TACI mutations fail to secrete IgG and IgA in response to APRIL *in vitro* but secrete normal amounts of IgG when stimulated by anti-CD40 plus IL-4.⁹ Similar results have been observed in B cells from TACI^{-/-} mice.¹ These results suggest that CD40-induced immunoglobulin secretion *in vitro* is independent of TACI. Conversely, TACI induction of immunoglobulin secretion *in vitro* is independent of CD40 because it occurs normally in CD40-deficient B cells.¹ Taken together, these data suggest that the CD40 and TACI pathways might activate CSR and immunoglobulin secretion in B cells independently of each other. Nevertheless, despite intact CD40 signaling, patients with CVID are impaired in their ability to respond to T-dependent antigens and to generate plasma cells,¹² raising the possibility that the CD40 and TACI pathways might intersect. In this study we demonstrate that these 2 pathways cooperate to promote B-cell differentiation into plasma cells and increase immunoglobulin production.

METHODS**Mice**

BALB/c mice were purchased from the Jackson Laboratory. B-cell maturation antigen (BCMA)^{-/-} mice and genetically matched (C57/BL6-129Sv) wild-type (WT) control animals were previously described.¹³ All mice were kept in a specific pathogen-free animal facility. All procedures performed on the animals were in accordance with the Animal Care and Use Committee of the Children's Hospital, Boston.

***In vitro* immunoglobulin production**

Naive B cells from BALB/c, WT, and BCMA^{-/-} mice were negatively sorted by using a cocktail of biotin-conjugated mAbs to CD43, CD11b, Thy1.2, CD138, IgG₁, IgG_{2a}, IgG_{2b}, IgG₃, IgA, and

IgE and streptavidin magnetic beads (Dyna, Oslo, Norway). B cells were cultured at 1×10^6 /mL in RPMI containing 10% FCS, L-glutamine, and 50 mmol/L 2-mercaptoethanol (complete medium). For immunoglobulin synthesis, B cells were cultured in one or more of the following: complete medium alone, IL-4 at 50 ng/mL (R&D Systems, Minneapolis, Minn), suboptimal concentration of anti-CD40 mAb (75 ng/mL; BD PharMingen, Franklin Lakes, NJ), APRIL at 1 μ g/mL, plate-bound polyclonal goat anti-TACI at 5 μ g/mL or goat IgG at 5 μ g/mL (all R&D Systems), and LPS at 10 μ g/mL (Sigma, St Louis, Mo). Cells were treated with 5 μ g/mL polymyxin B (Sigma), except when LPS was added. After 6 days, supernatants were assayed for IgG₁ and IgE by means of ELISA.¹⁴

RT-PCR for germ-line transcripts, AICDA, and postswitch (I μ -C μ) transcripts

RNA was extracted from 3-day cultured naive B cells by using TRIzol and was reverse transcribed with Superscript II RT (all Invitrogen, Carlsbad, Calif). The PCR primers used, I μ , I γ ₁, C γ ₁, I ϵ , C ϵ , AICDA, and β ₂-microglobulin, were previously described.^{14,15} All PCR reactions were performed on 1:1, 1:3, and 1:9 cDNA dilutions for semiquantitative evaluation. Amplified products were separated on agarose gel and stained with ethidium bromide.

Staining and carboxyfluorescein succinimidyl ester assay

Naive B cells from BALB/c mice stimulated with anti-CD40 for 72 hours were stained with anti-TACI-PE (phycoerythrin, R&D Systems) or anti-BCMA-fluorescein isothiocyanate (R&D Systems) or were stimulated for 6 days as indicated and then stained with anti-CD138-phycoerythrin (PE), anti-IgG₁-PE, or both or biotin conjugated (BD PharMingen), followed by streptavidin-allophycocyanine (eBioscience, San Diego, Calif). For carboxyfluorescein succinimidyl ester (CFSE) assays, naive B cells were loaded with 10 mmol/L CFSE (Molecular Probes, Eugene, Ore) and then stimulated and stained as above. For survival assays, B cells were stimulated as indicated and stained every day for 6 days with Annexin V-fluorescein isothiocyanate (BioVision, Lyon, France). All staining was analyzed by means of FACS.

Quantitative PCR

cDNA obtained as described above was used for real-time PCR reactions and run on an ABI Prism 7300 (Applied Biosystems, Foster City, Calif). Tagman primers with 6-carboxyfluorescein-labeled probes for Blimp1, IRF4, IL6, and the control β ₂-microglobulin were obtained from Applied Biosystems. Relative gene expression among the different samples was calculated as described by Pfaffl.¹⁶

Statistics

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

RESULTS**APRIL increases immunoglobulin production induced by anti-CD40 and IL-4 by engaging TACI**

To test the hypothesis that CD40-induced immunoglobulin production is increased by engagement of TACI, we first examined the effect of TACI ligation on immunoglobulin synthesis induced by a suboptimal concentration of anti-CD40 mAb in negatively sorted naive splenic B cells from BALB/c mice. B cells were stimulated

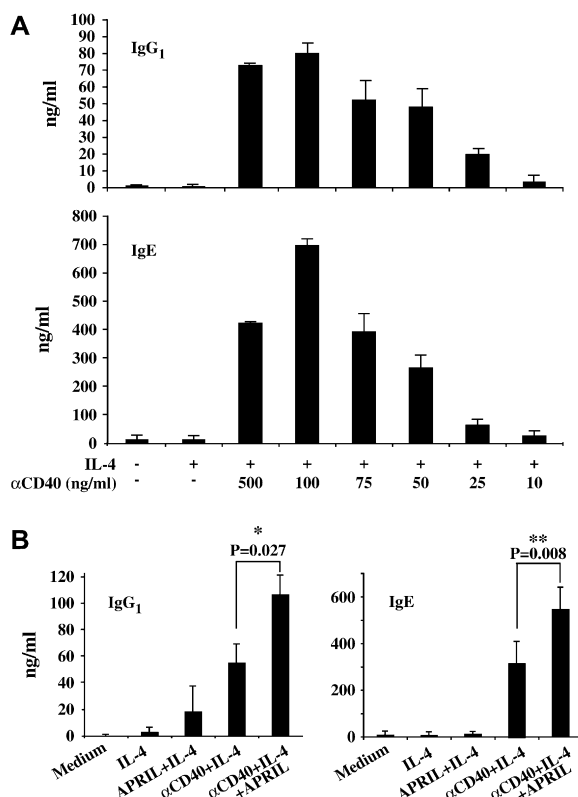


FIG 1. Effect of APRIL on IgG₁ and IgE production induced by anti-CD40 and IL-4. **A**, IgG₁ and IgE production by naive B cells in response to different concentrations of anti-CD40 and IL-4. **B**, Effect of APRIL on IgG₁ and IgE production driven by a suboptimal concentration of anti-CD40 (75 ng/mL). Bars represent the mean $\pm$ SE of 3 (Fig 1, A) and 4 (Fig 1, B) independent experiments.

in vitro with concentrations of anti-CD40 ranging from 500 to 10 ng/mL and IL-4 to determine the suboptimal concentration of anti-CD40 for our experiments. Fig 1, A, shows that 100 ng/mL anti-CD40 consistently achieved optimal induction of IgG₁ and IgE synthesis, whereas a concentration of 75 ng/mL anti-CD40 resulted in just suboptimal synthesis of IgG₁ and IgE. Subsequently, we used this concentration to examine the effect of TACI ligation on CD40-driven IgG₁ and IgE synthesis. Fig 1, B, shows that addition of APRIL caused a significant approximately 2.5-fold increase of IgG₁ and IgE secretion by B cells treated with IL-4 and 75 ng/mL anti-CD40. APRIL plus IL-4 induced modest IgG₁ and IgE synthesis, as previously described.¹

APRIL binds to BCMA and heparan sulfate proteoglycans (HSPGs) in addition to TACI.^{17,18} Either of these receptors could potentially mediate the enhancing effect of APRIL on CD40-driven immunoglobulin production by naive B cells. Fig 2 shows that naive B cells express TACI but no detectable BCMA. CD40 ligation upregulated TACI expression but had no detectable effect on BCMA expression after 72 hours. This finding suggests that the effect of APRIL on CD40 activation of B cells was mediated through TACI.

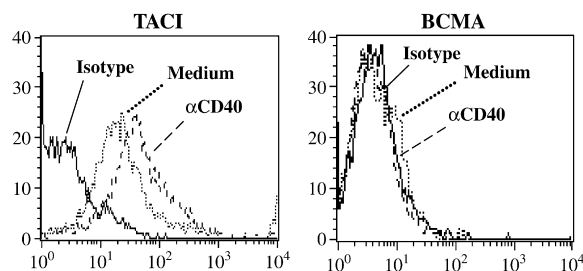


FIG 2. Effect of CD40 ligation on TACI expression. FACS analysis of TACI and BCMA expression in naive mouse B cells before and after stimulation with anti-CD40 for 72 hours is shown. Similar results were obtained in 3 independent experiments.

To ascertain the role of TACI in APRIL-mediated enhancement of CD40 activation, we examined the effect of anti-TACI antibody. Fig 3, A, shows that addition of goat anti-TACI enhanced CD40 plus IL-4-driven IgG₁ and IgE production by naive B cells to a similar extent as APRIL. In contrast, control goat IgG had a negligible effect. Like APRIL, anti-TACI, in the presence of IL-4, induced IgG₁ and IgE production (a net increase of 30.3 ng/mL for IgG₁ and 43 ng/mL for IgE), confirming our previous finding that engagement of TACI causes CSR in naive B cells.

To exclude an autocrine mechanism by which engagement of TACI might induce expression of APRIL, which in turn might engage BCMA and HSPGs, we tested the ability of anti-TACI to increase immunoglobulin production induced by anti-CD40 plus IL-4 in B cells from BCMA^{-/-} mice. In all experiments B cells from WT control and BCMA^{-/-} mice treated with anti-CD40 plus IL-4 plus anti-TACI secreted higher amounts of IgG₁ and IgE than B cells treated with anti-CD40 plus IL-4 plus control goat IgG. We observed variability between experiments in the amounts of IgG₁ and IgE produced by B cells from both BCMA^{-/-} and WT control animals, possibly because of the mixed background of these mice (C57/B16-129Sv). For this reason, we have expressed the data as the fold increase in immunoglobulin secretion. There was no significant difference in the increase of IgG₁ and IgE synthesis induced by TACI ligation between BCMA^{-/-} mice and WT control animals (Fig 3, B). These results suggest that TACI mediates APRIL enhancement of immunoglobulin production induced by CD40. In addition, the use of anti-TACI eliminates the potential contribution of BCMA or HSPGs that can occur with the use of APRIL.

TACI engagement does not increase B-cell proliferation and survival induced by anti-CD40 and IL-4

CD40-driven CSR is thought to be division linked.¹⁹ We tested the hypothesis that engagement of TACI increases the rate of division of CD40/IL-4-activated B cells. CFSE dye dilution analysis revealed that B cells stimulated with anti-CD40 plus IL-4 underwent 8 division

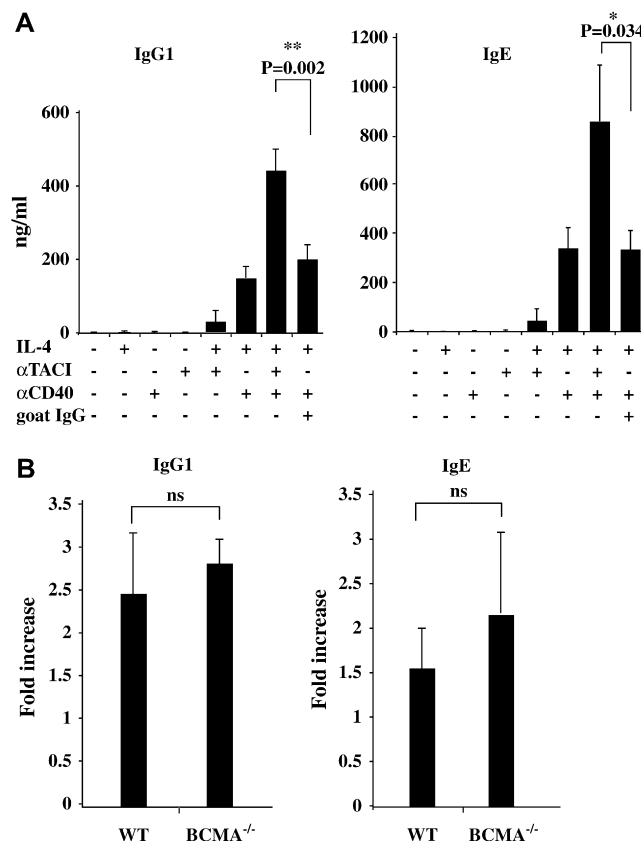


FIG 3. Effect of TACI ligation on IgG₁ and IgE production induced by anti-CD40 and IL-4. Effect of anti-TACI on IgG₁ or IgE production in B cells from BALB/c (A) mice and from BCMA^{-/-} mice and WT control animals (B) is shown. Bars represent the mean $\pm$ SE of 5 independent experiments in Fig 3, A, and 4 independent experiments in Fig 3, B.

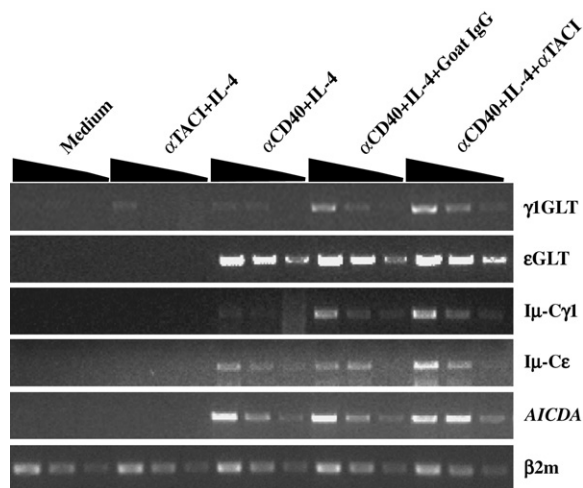


FIG 4. Effect of TACI ligation on molecular events involved in CSR induced by anti-CD40 plus IL-4. B cells were cultured for 3 days with the indicated stimuli. PCR was performed on cDNA samples diluted 1:1, 1:3, and 1:9. β_2 -Microglobulin was used as a loading control. Similar results were obtained in 3 independent experiments.

cycles in the presence of anti-TACI antibody versus 9 cycles in the presence of control IgG (see Fig E1, A, in the Online Repository at <http://www.jacionline.org>).

We also examined the effect of anti-TACI on the survival of B cells stimulated with anti-CD40. We used Annexin V staining to detect apoptotic cells at days 1, 2, 3, 4, and 6 after stimulation. As expected, the percentage of live cells was substantially higher in cells stimulated with anti-CD40 plus IL-4 compared with those stimulated with medium (see Fig E1, B, in the Online Repository at <http://www.jacionline.org>). Addition of anti-TACI did not significantly affect the percentage or number of live cells. Altogether, these data suggest that the increase in immunoglobulin production observed when both TACI and CD40 are engaged is neither due to clonal expansion nor to improved B-cell survival.

TACI engagement has little effect on the induction of γ_1 germ-line transcripts, ϵ germ-line transcript, $I\mu$ -C γ_1 , $I\mu$ -C ϵ , and activation-induced cytidine deaminase by anti-CD40 and IL-4

Molecular events involved in CSR include expression of germ-line transcripts (GLTs) and *AICDA*, followed by deletional switch recombination and expression of $I\mu$ -C γ_1 mature transcripts.²⁰ We tested the possibility that TACI signaling cooperates with CD40 and IL-4 signaling in inducing molecular events leading to CSR. As expected,

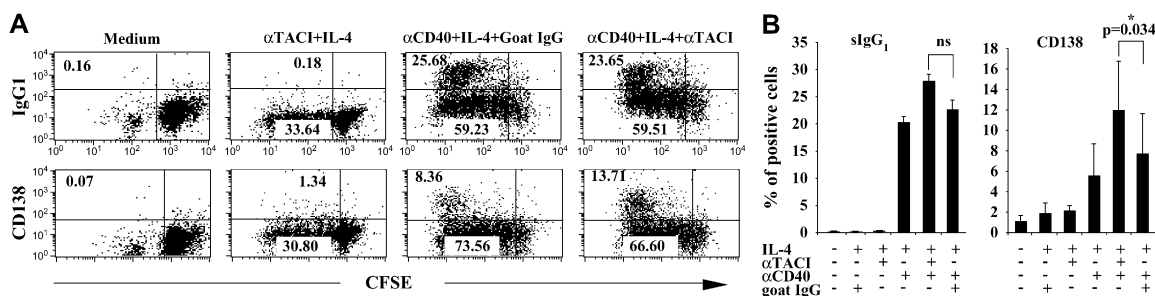


FIG 5. Effect of TACI ligation on IgG₁ and CD138 surface expression induced by anti-CD40 plus IL-4. **A**, B cells were loaded with CFSE, cultured for 6 days, and then analyzed by using FACS for IgG₁ and CD138 surface expression. **B**, Pooled results of 5 independent experiments. Bars represent the mean ± SE.

anti-CD40 plus IL-4 induced γ_1 GLT, ϵ GLT, and *AICDA* gene expression. There was only a slight increase in γ_1 GLT, ϵ GLT, $I\mu$ -C γ_1 , $I\mu$ -C ϵ , and *AICDA* mRNA expression on addition of anti-TACI compared with goat IgG (Fig 4). Note that control goat IgG resulted in a non-specific increase in the γ_1 GLT and $I\mu$ -C γ_1 signals.

TACI engagement increases the percentage of CD138⁺, but not of surface IgG₁⁺, cells induced by anti-CD40 and IL-4

We next examined whether the increase in IgG₁ secretion induced by TACI ligation of naive B cells stimulated with anti-CD40 plus IL-4 was associated with increased induction of surface (s)IgG₁⁺ cells. Fig 5, A (upper panels) and B (left panel), shows the results of a representative experiment and pooled results from 5 independent experiments in which unlabeled or CFSE-labeled B cells were stained at day 6 for sIgG₁ expression. Addition of anti-TACI to cultures of B cells stimulated with anti-CD40 plus IL-4 caused no significant increase in sIgG₁⁺ B cells compared with addition of control goat IgG. Anti-TACI plus IL-4 caused no detectable increase in sIgG₁ expression, possibly because TACI by itself is a relatively weak inducer of IgG₁ switching. These results suggest that the addition of anti-TACI to anti-CD40 has a negligible effect on CSR to IgG₁.

In vitro stimulation with anti-CD40 plus IL-4 induces naive B cells to differentiate into plasmablasts that secrete immunoglobulins in the culture supernatant and express on their surface syndecan-1/CD138. Fig 5, A (lower panels) and B (right panel), shows the results of experiments in which the same B cells used to analyze sIgG₁ expression were examined for CD138 expression 6 days after stimulation. There was robust induction of CD138 expression in cultures stimulated with anti-CD40 plus IL-4 but little induction of CD138 expression by anti-TACI plus IL-4, again possibly because TACI is a relatively weak inducer of isotype switching. Addition of anti-TACI, but not control goat IgG, to cultures stimulated with anti-CD40 plus IL-4 caused a significant increase in the percentage of CD138⁺ cells compared with control goat IgG (13.71% vs 8.36%, $P = .034$).

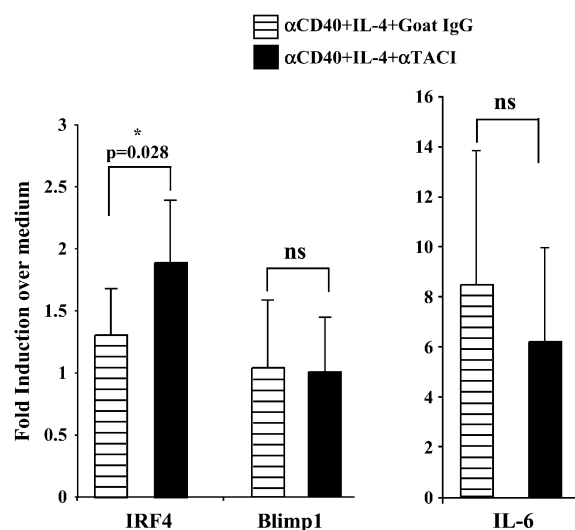


FIG 6. Effect of TACI ligation on the induction of IRF4, Blimp1, and IL-6 expression by anti-CD40 plus IL-4. Real-time PCR on RNA from B cells cultured for 3 days with anti-D40 plus IL-4 and either goat IgG or goat anti-TACI. Bars represent the mean ± SE of 4 independent experiments.

TACI engagement causes a modest increase in IRF4 expression in B cells stimulated with anti-CD40 plus IL-4

We examined the effect of anti-TACI on the expression of the plasma cell differentiation factors Blimp1 and IRF4 and on the expression of the cytokine IL-6, which delivers survival signals to bone marrow plasma cells.²¹ Fig 6 shows that stimulation with anti-CD40 plus IL-4 and goat IgG for 3 days slightly increased IRF4 expression in naive B cells and caused no detectable increase in Blimp1 expression. The same B cells strongly upregulated Blimp1 expression after LPS stimulation (43.9 ± 27.2 -fold induction, $n = 3$). Addition of anti-TACI to anti-CD40 plus IL-4 caused a significant ($P = .028$) increase in IRF4 mRNA expression compared with control goat IgG. In contrast, Blimp1 expression remained unaffected. Anti-TACI plus IL-4 did not significantly upregulate either IRF4 or Blimp1 expression (data not shown). IL6 mRNA expression was strongly upregulated by anti-CD40 plus IL-4.

Addition of anti-TACI caused no further increase in IL6 expression.

DISCUSSION

Our data indicate that TACI enhances the differentiation of B cells activated under limiting conditions of CD40 ligation into immunoglobulin-secreting plasmablasts. This suggests that in addition to its key role in the antibody response to T-independent antigens, TACI might be important for the antibody response to T-dependent antigens.

We demonstrated that APRIL and anti-TACI enhanced IgG₁ and IgE synthesis by naive B cells stimulated with suboptimal concentrations of anti-CD40 and IL-4. In turn, CD40 stimulation upregulated TACI expression on B cells. Thus there is crosstalk between TACI and CD40 because TACI ligation enhances immunoglobulin production induced by CD40 and CD40 ligation induces increased expression of TACI on the surface of B cells. Recently, it was reported that engagement of HSPG is important for APRIL-induced B-cell proliferation and production of IgA.²² It is possible that TACI induction of CSR to IgA might have different signaling requirements than TACI enhancement of CD40-driven B-cell activation.

Among the functions that we analyzed, TACI and CD40 cooperation is limited to the immunoglobulin secretion. There was only a modest increase in the induction of the molecular events underlying CSR and no significant increase in the generation of sIgG₁⁺ switched B cells. Moreover, CD40-induced proliferation and viability of B cells was not affected by TACI engagement. In contrast, there was an increase in the percentage and number of CD138⁺ plasmablasts that correlated with the increase in immunoglobulin secretion. These results suggest that TACI ligation exerted its effect on CD40-stimulated B cells after switching by inducing more B cells to differentiate to plasmablasts, possibly by enhancing the survival of plasmablasts, or both. Although TACI appears to promote the generation of plasmablasts, BCMA is thought to be important for the survival of long-lived bone marrow plasma cells but is not implicated in the generation of short-lived plasma cells.²³ Thus TACI and BCMA might cooperate in inducing optimal immunoglobulin secretion.

The transcription factors Blimp1 and IRF4 play a critical role in plasma cell differentiation.^{4,5,24} Although TACI ligation caused a robust increase in the generation of plasmablasts after CD40/IL-4 stimulation, it caused a modest, albeit significant, increase in IRF4 expression and no detectable increase in Blimp1 expression. This suggests that TACI might activate pathways in addition to IRF4 and other than Blimp1 that promote plasma cell generation, survival, or both. Potential candidates include Mcl-1, which is induced by TACI ligation and promotes the survival of B-cell blasts and plasmablasts.²⁵ Alternatively, the modest increase observed in the levels of IRF4 might be sufficient to promote plasma cell differentiation.

The synergy we have observed between TACI ligation and CD40 might involve signaling pathways common to both receptors. These include recruitment of TNF receptor-associated factor molecules and activation of mitogen-activated protein kinases and the transcription factors nuclear factor κ B and activator protein 1,^{26,27} which have been shown to be important for CD40-induced CSR.^{14,28} In particular, TNF receptor-associated factor 6, which is engaged by both CD40 and TACI, is thought to be important for plasma cell differentiation.²⁹ Alternatively, TACI might recruit specific signaling intermediates, such as calcium modulator and cyclophilin ligand, that synergize with pathways activated by CD40 to promote B-cell differentiation.

Sakurai et al³⁰ recently reported that agonistic anti-TACI mAb attenuates proliferation and IgG secretion by human B cells activated by a relatively high concentration of sCD40 ligand (2 mg/mL). This suggests that TACI ligation might have opposite effects on CD40 activation of B cells depending on the strength of the CD40 signal. These opposite effects could be mediated by prostaglandin (PG) E₂, the synthesis of which is induced by TACI ligation.²⁵ Low concentrations of PGE₂ enhance B cell-differentiation and immunoglobulin production, whereas high concentrations of PGE₂ are inhibitory.²⁵ CD40 ligand expression has been reported to be decreased in patients with CVID.³¹ Our data suggest that impairment of TACI function could also contribute to the impaired antibody response to T-dependent antigens in these patients. Although it has been reported that TACI^{-/-} mice mount a normal antibody response when immunized with T-dependent antigens by using standard protocols,³² our data suggest that the response of these mice to suboptimal doses of T-dependent antigen needs to be investigated.

GCs are a candidate location where B cells might receive signals through CD40, TACI, and the B-cell receptor. In GCs B cells are in contact both with activated T cells that express CD40 ligand and with dendritic cells that express APRIL and B cell-activating factor of the TNF family. Furthermore, ligation of B-cell receptor, CD40, and TLR9 upregulates TACI expression on B cells (Fig 2).³³ TACI upregulation would be expected to further promote cooperation between TACI and CD40 in the response to T-dependent antigens.

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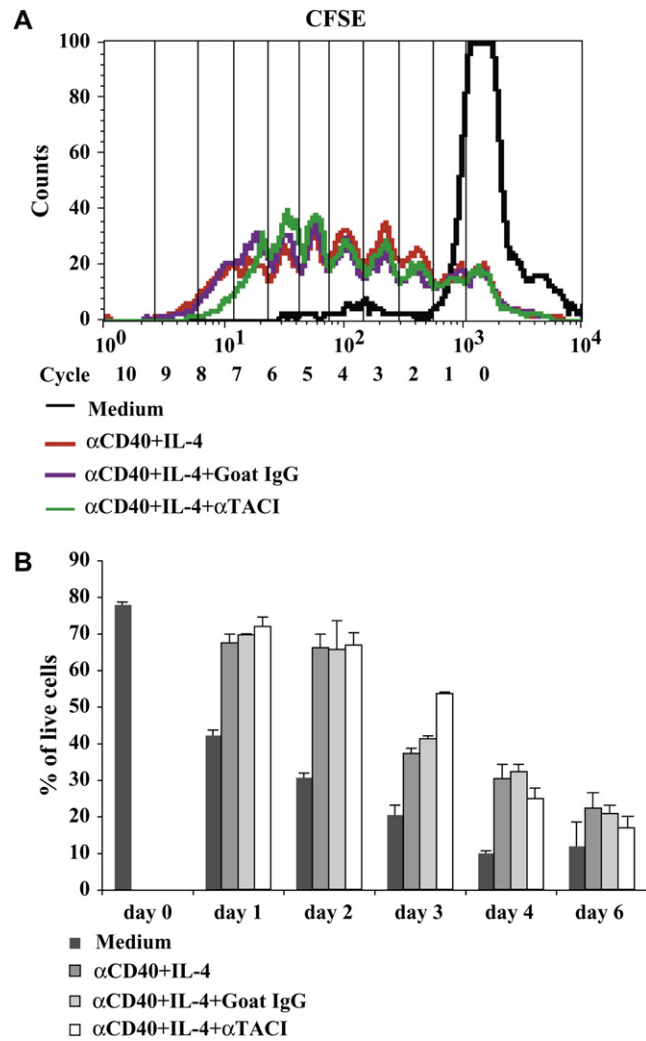


FIG E1. Effect of TACI ligation on B-cell proliferation and survival in cultures stimulated with anti-CD40 plus IL-4. **A**, B cells were loaded with CFSE dye at day 0 and analyzed by using FACS at day 6 after stimulation with medium (black line), anti-CD40 plus IL-4 (red line), anti-CD40 plus IL-4 plus control goat IgG (purple line), and anti-CD40 plus IL-4 plus anti-TACI (green line). Similar results were obtained in 2 other independent experiments. **B**, B cells stimulated as above were stained with Annexin V–fluorescein isothiocyanate and analyzed by using FACS immediately after purification and at days 1, 2, 3, 4, and 6 of culture. Bars represent the mean $\pm$ SE of 3 independent experiments.