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TLR9, TACI and CD40 synergize in causing B cell activation

Esra Ozcan, MD¹, Ingrid Rauter, PhD¹, Lilit Garibyan, MD, PhD¹, Stacey R. Dillon, PhD², and Raif S. Geha, MD^{1,*}

¹Division of Immunology, Children's Hospital and Department of Pediatrics, Harvard Medical School, Boston, MA, 02115

²ZymoGenetics, Inc., Seattle, WA

Abstract

Background—B cells receive activating signals from T cells via CD40, from microbial DNA via TLR9, and from dendritic cells (DCs) via transmembrane activator calcium modulator and 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—To examine the mechanisms by which TLR9 modulates CD40- and TACI-mediated activation of B cells, and to determine whether all three receptors synergize to activate B cells.

Methods—Naïve mouse B cells and human peripheral blood mononuclear cells (PBMCs) were stimulated with combinations of anti-CD40, CpG and APRIL in the presence of IL-4. Proliferation was measured by ³H-thymidine incorporation. Immunoglobulin production was measured by ELISA. Class switch recombination (CSR) was examined by measuring mRNA for germ line transcripts, activation-induced cytidine deaminase (*AICDA*) and mature Ig transcripts. Plasma cell differentiation was examined by 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 naïve murine B cells, and synergized with TACI in driving B cell proliferation and plasma cell differentiation. All three receptors synergized together in driving mouse 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.

Clinical Implications—Stimulation of B cells via CD40, TLR9 and TACI results in their optimal activation. Defects in these pathways may co-operate to impair B cell immunoglobulin production in patients with common variable immunodeficiency (CVID).

Keywords

TLR9; CD40; TACI; CSR; plasma cell; immunoglobulin; B cells

*Corresponding author: Raif S. Geha, Division of Immunology, Children's Hospital, One Blackfan Circle, Boston, Massachusetts 02115, USA. Tel. (617) 919 2482; Fax: (617) 730 0528; raif.geha@childrens.harvard.edu.

INTRODUCTION

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, BAFF/APRIL-TACI and TLR ligands-TLRs¹⁻³.

B cell activation by CD40 stimulation is essential for T cell dependent (TD) antibody responses and is the major contributor to immunoglobulin production *in vivo*. This is evidenced by the observation that mice and patients deficient in CD40L or CD40 have severely decreased levels of serum IgG, lack serum IgE, fail to mount antigen specific antibody responses following immunization with TD 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 (B cell activating factor belonging to TNF superfamily) and APRIL (a proliferation inducing ligand)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 cells⁹. 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, deficient antibody responses to the Type II T-independent (TI) antigens Pneumovax and TNP-Ficoll¹³⁻¹⁶. Furthermore, they have an impaired antibody response to immunization with a suboptimal dose of the Type I TI antigen TNP-LPS, indicating that TACI synergizes with TLR4 *in vivo*¹². Heterozygous mutations in TACI are found in ~10% of common variable immune deficiency (CVID) patients and in ~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 ligands²³.

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 may be exposed to exogenous and endogenous TLR ligands released by microbes and dying B cells in GCs. Thus, B cells responding to antigen may simultaneously receive signals via CD40, TACI and TLR9. These three receptors may synergize in pair or all together 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 naïve B cells¹¹. 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 is contradictory²⁵⁻²⁷. 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 via CD40, TLR9, and TACI synergize to increase immunoglobulin production by mouse naïve 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 normal subjects.

MATERIALS & METHODS

Mice

BALB/c mice were purchased from Charles River Laboratories. TACI^{-/-}, BCMA^{-/-} and genetically matched wild-type (WT) mice on 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 Animal Care and Use Committee of the Children's Hospital, Boston.

Immunoglobulin Production and Proliferation by Mouse B Cells

Naïve B cells were negatively sorted from mouse splenocytes using a cocktail of biotin-conjugated mAbs to CD43, CD11b, Thy1.2, CD138, IgG1, IgG2a/b, IgG3, IgA and IgE, and streptavidin magnetic beads (Dynabeads M-280, Invitrogen). 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. Naïve B cells were stimulated with APRIL at 1 µg/ml (R&D Systems), IL-4 at 50 ng/ml (R&D Systems), CpG oligonucleotide 1826 (InvivoGen, California) at 10 ng/ml, 100 ng/ml or 3 µg/ml, anti-CD40 antibody (BD Pharmingen) at 1 ng/ml, 10 ng/ml 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 described²⁹. After 72 hours, cultures were pulsed with 1 µCi ³H-thymidine for an additional 16 hours then harvested, and scintillation counted.

Surface Staining

B cells were stained on day 6 after stimulation with anti-B220-FITC, antiCD138-PE or anti-IgG1-PE (BD Pharmingen) and analyzed by FACS (BD FacsCalibur), as previously described¹¹.

B cell Survival

B cells were stained on day 3 after stimulation with Annexin V-fluorescein isothiocyanate and propidium iodide (Bio Vision) and analyzed by FACS.

RT-PCR Analysis

RNA extracted from 4-day cultures using TRIzol (Invitrogen) was reverse transcribed by Superscript II RT (Invitrogen). PCR primers and conditions used to detect Iε-Cε, Iµ-Cε, Iγ1-Cγ1, Iµ-Cγ1, *AICDA* and β2 microglobulin were previously described²⁹. Amplified products were separated on agarose gels and stained with ethidium bromide.

Q-PCR Analysis

Real-time PCR reaction was run on cDNA using ABI Prism 7300 (Applied Biosystems). Taqman primers for 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 µM), zzAPRIL (10 nM, ZymoGenetics, Inc.), 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) for 14 days³⁰. Supernatants were harvested and IgG was assayed by ELISA. Net IgG synthesis was calculated by subtracting the amount of IgG in unstimulated cultures.

Statistics

p values were calculated using the paired *t* test with PRISM software (Prism Software Corp).

RESULTS

CpG synergizes with CD40 ligation in driving immunoglobulin (Ig) production and proliferation by naïve B cells

A range of concentrations of anti-CD40 (1 to 100 ng/ml) and CpG (10 ng/ml to 3 µg/ml) were tested for their ability to drive IgG1 and IgE production by negatively sorted naïve B cells (95% IgM⁺IgD⁺ cells) from BALB/c mice, in the presence of IL-4. Based on the results obtained (Online repository material, Figures 1 and 2), 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 naïve B cells. Figure 1A 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 to those secreted by B cells stimulated with an optimal concentration of 100 ng/ml anti-CD40+IL-4. There was no detectable synergy between CpG and anti-CD40 in driving IgM production (data not shown).

IgG1 and IgE production by naïve B cells involves isotype switching, which is thought to be linked to B cell proliferation³¹. Figure 1B shows that stimulation with suboptimal concentrations of CpG and anti-CD40 had an additive effect on the proliferation of naïve 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+IL-4, but was significantly less than that induced by 3 µg/ml CpG+IL-4. Staining with Annexin V and propidium iodide (PI) revealed no detectable effect of CpG (100 ng/ml) on the survival of naïve B cells stimulated with 10 ng/ml anti-CD40+IL-4, as determined by the percentage of Annexin V⁺ PI⁺ cells (Fig. 1C).

CpG synergizes with CD40 ligation in driving isotype switching

To investigate potential synergy of TLR9 and CD40 in driving CSR, we examined surface IgG1 (sIgG1) expression by naïve B cells stimulated for 6 days with suboptimal concentrations of CpG and anti-CD40 in the presence of IL-4. Figure 2A 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 to those in cultures stimulated with optimal concentrations of CpG (3 µg/ml)+IL-4 or anti-CD40 (100 ng/ml)+IL-4.

CSR requires expression of germ line transcripts (GLTs) and *AICDA* followed by deletional switch recombination and expression of post-switch mature transcripts (I μ -C H)³². Fig. 2B 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* (activation induced cytidine deaminase), I μ -C ϵ and I μ -C γ 1 mature transcripts as determined by 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 to cultures stimulated with either (Fig. 2C and D). These results suggest that the synergy between TLR9 and CD40 is exerted primarily at the level of B cell proliferation and CSR. It should be noted that there was ten-fold less IgG1 secretion by B cells stimulated with optimal concentrations of CpG than with optimal concentrations of anti-CD40 with despite comparable expression of sIgG1, *Blimp-1* mRNA

and CD138. While clearly, sIgG1 expression need not correlate with IgG1 secretion. There is also data to suggest that neither *Blimp-1* expression nor expression of plasmablast markers is sufficient for a high rate of Ig secretion ³³.

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

Pre-incubation 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 naïve B cells (Online repository material, Fig. 3A and data not shown). CpG and APRIL also synergized in driving IgG1 and IgE secretion by naïve B cells in the presence of IL-4 (Fig. 3A) to levels comparable to those observed with optimal amounts of CpG (3 µg/ml)+IL-4. The ten-fold lower IgG1 and IgE secretion by B cells stimulated with APRIL +CpG compared to B cells stimulated with anti-CD40+CpG at the same concentration reflects the fact that anti-CD40 even at a concentration of 10ng/ml is a more potent B cell stimulator than APRIL. CpG synergized with APRIL in driving the proliferation of naïve B cells in the presence of IL-4 (Fig. 3B) and in its absence (Online repository material Fig. 3B). There was no detectable effect of CpG on the survival of naïve B cells stimulated with APRIL+IL-4 (Fig. 3C) or APRIL alone (Online repository material Fig. 3C).

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

APRIL binds to TACI, BCMA and heparan sulfate proteoglycans (HSPGs) on B cells ^{8, 34}. Synergy between CpG and APRIL in driving IgG1 and IgE production in B cells stimulated with IL-4 stimulated was strictly dependent on TACI, as it was preserved in B cells from BCMA^{-/-} mice, but not in B cells from TACI^{-/-} mice (Online repository material Fig. 4).

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

We next tested for potential triple synergy between TLR9, CD40 and TACI in B cell activation. Suboptimal concentrations of CpG and anti-CD40 synergized with APRIL in driving IgG1 and IgE secretion and proliferation of naïve B cells cultured with IL-4 (Fig. 5A and B). The ten-fold lower IgG1 and IgE secreted by B cells stimulated with all three ligands compared to B cells stimulated with anti-CD40+CpG is likely due to the fact that we used a ten-fold lower concentration of anti-CD40 in the triple synergy than in the double synergy experiment (1 versus 10 ng/ml). There was no detectable synergy between the three 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 Cε GLT, *AICDA*, Iµ-Cε and Iµ-Cγ1 mature transcripts (Fig. 5C and D). Cγ1 GLT expression was not affected. The three ligands were additive inducing the expression of CD138 on naïve B cells cultured with IL-4 (Fig. 5E), and synergized in inducing mRNA expression of *Blimp-1* by these cells (Fig. 5F).

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 humans. Based on preliminary dose response

experiments, we stimulated PBMCs from adult normal subjects with suboptimal concentrations of CpG at 0.001 μ M, zz-APRIL at 10 nM, and anti-CD40 mAb at 10 ng/mL. All cultures received IL-4 (5 ng/mL). CpG synergized both with anti-CD40 and with zzAPRIL in driving IgG secretion by human PBMCs (Fig. 6).

DISCUSSION

The present work demonstrates that TLR9, CD40 and TACI 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 in naïve mouse B cells cultured in the presence of IL-4 (Fig. 1A). Simultaneous ligation of TLR9 and CD40 had additive effects on B cell proliferation (Fig. 1B) and synergized in driving CSR, as evidenced by synergistic induction of sIgG1 expression, C ϵ and C γ 1 germ line transcription, *AICDA* expression and expression of mature IgG1 and IgE transcripts (Fig. 2A,B). There was no detectable synergy exerted on plasma cell differentiation, as measured by expression of surface CD138 and Blimp-1 mRNA (Fig. 2C,D). Thus, CSR is a major target for the synergy between CD40 and TLR9. TLR9 upregulates CD40 expression on human and mouse B cells^{37–39}. However, in contrast to what has been reported in mouse macrophages⁴⁰, CD40 ligation did not upregulate TLR9 mRNA expression in mouse B cells (data not shown). Enhanced signaling due to 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 cells²⁶. However, CpG was reported to inhibit CD40/IL4 driven IgE synthesis in human B cells and IgG1 and IgE synthesis in mouse B cells^{25–27}. The discrepancy between these results and ours may be related to the use of different concentrations of ligands. Previous mouse studies used concentrations of CpG (3 μ M, equivalent to 20 μ g/ml) and CD40 (3 and 10 μ g/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 may differ resulting in opposite effects on B cell activation. The previous studies on human B cells have used CD40L-transfected CHO cells²⁶, making it difficult to determine the effective concentration of CD40L. In the present work, we have 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 suggests that synergy would occur when these concentrations are suboptimal.

TLR9 synergized with TACI in driving IgG1 and IgE secretion proliferation (Fig. 3A,B) and CSR (Fig. 4A,B) in IL-4 stimulated naïve B cells and exerted additive effects on plasma cell differentiation, as measured by the expression of surface CD138 and Blimp-1 mRNA levels (Fig. 4C, D). Thus 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 mouse B cells^{21, 41}. 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 all 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 three receptors to achieve optimal activation and antibody production. These may include NFκB, activated by all three receptors^{19, 42, 43}, NF-AT activated by TACI⁴³, and STAT3 activated by TLR9⁴⁴. NFκB is critical for *AICDA* induction⁴⁵, and STAT3 is critical for *Blimp-1* expression and plasma cell differentiation^{46, 47}.

Activated T cells and follicular DCs are well-established sources of CD40 and TACI ligands respectively in GCs^{48, 49}. Mitochondrial DNA, released by damaged tissue, has been shown to engage TLR9 in neutrophils⁵⁰, 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 naïve B cells in order to study the cellular and molecular events which are involved in B cell differentiation from naïve cells to plasmablasts, synergy between CD40 TACI and TLR9 is likely to operate not only in naïve 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 naïve 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 three 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^{35, 36}. 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 co-operate to impair B cell immunoglobulin production in CVID.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AICDA	Activation induced cytidine deaminase
APRIL	a proliferation inducing ligand
BAFF	B cell activating factor belonging to TNF superfamily
Blimp-1	B-lymphocyte-induced maturation protein 1
CSR	Class switch recombination
CVID	common variable immune deficiency
DCs	Dendritic cells
GC	Germinal Center
GLTs	Germ line transcripts

Ig	Immunoglobulin
PBMCs	Peripheral blood mononuclear cells
PI	Propidium iodide
TACI	transmembrane activator calcium modulator and cyclophilin ligand interactor
TD	T cell dependent
TI	T-independent
WT	wild-type

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A **B** **C**

Figure 1. TLR9 synergizes with CD40 in activating naïve B cells.

A, B Synergy between CpG and anti-CD40 in driving IgG1 and IgE production (A), and proliferation (B) in naïve B cells cultured with IL-4. C. Effect of addition of CpG to anti-CD40 mAb on B cell survival. Values represent the mean $\pm$ SD of at least 3 experiments.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Note that in this and subsequent figures, not all bars representing SDs are readily visible.

Figure 2. TLR9 synergizes with CD40 in driving isotype switching

A. sIgG1 expression. **B.** Expression of CεGLT, Iμ-Cε, Cγ1GLT, Iμ-Cγ1 and *AICDA* mRNA measured by RT-PCR. Similar results were obtained in 3 independent experiments. **C.** CD138 surface expression. **D.** Blimp-1 mRNA expression. Values in A, C and D represent the mean±SD of at least 3 experiments. ***p<0.001.

Figure 3. TLR9 synergizes with TACI in driving immunoglobulin (Ig) production and proliferation

A, B. Synergy between CpG and APRIL in driving IgG1 and IgE production (**A**), and proliferation (**B**) in naïve B cells stimulated with IL-4. **C.** Effect of addition of CpG to APRIL on B cell survival. Values represent the mean±SD of at least 3 experiments.

*p<0.05, **p<0.01.

Figure 4. TLR9 and TACI synergize in driving isotype switching and have an additive effect on plasma cell differentiation

A. sIgG1 expression **B.** Expression of CεGLT, Iμ-Cε, Cγ1GLT, Iμ-Cγ1 and *AICDA* mRNA. Similar results were obtained in 3 independent experiments. **C.** CD138 surface expression **D.** Blimp-1 mRNA expression. Values in A, C and D represent the mean±SD of at least 3 experiments. *p<0.05, **p<0.01.

Figure 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), proliferation (B), sIgG1 expression (C), molecular events involved in CSR (D), CD138 expression (E) and Blimp-1 mRNA expression (F) by naïve B cells cultured with IL-4. Values in A, B, C, E and F represent the mean±SD of at least 3 experiments. D is representative 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001.

Figure 6. Synergy between CpG, anti-CD40 and APRIL in PBMCs from normal 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.