

Synthetic CpG oligodeoxynucleotides augment BAFF- and APRIL-mediated immunoglobulin secretion

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The B lymphocyte-activating factor belonging to TNF superfamily (BAFF) acts on B lymphocytes through BAFF receptor (BAFF-R), the transmembrane activator, calcium modulator, and cyclophilin ligand interactor (TACI), and the B cell maturation antigen (BCMA). Another cytokine, a proliferation-inducing ligand (APRIL), only binds to TACI and BCMA. In this study, we sought to determine the effect of Toll-like receptor agonists (TLR-A) on the expression of BAFF/APRIL receptors by murine splenic B lymphocytes. CpG oligodeoxynucleotides (ODN) and LPS strongly up-regulated TACI expression, while BAFF-R was only up-regulated by CpG ODN. CpG ODN pretreatment up-regulated TACI expression on follicular and marginal zone B lymphocytes and increased their responses to BAFF- and APRIL-mediated Ig secretion. TACI seemed to be playing a pivotal role in BAFF- or APRIL-induced Ig secretion because B lymphocytes from TACI-knockout mouse or the blocking of TACI with a neutralizing antibody resulted in total inhibition of IgA and IgG secretion in CpG ODN-pretreated and BAFF- or APRIL-stimulated B cells. Thus, CpG ODN-induced increase in TACI expression is likely to play an important role in Ig secretion following activation of B lymphocytes through TLR9.

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Abbreviations: **APRIL:** a proliferation-inducing ligand ·

BAFF: B lymphocyte-activating factor belonging to TNF superfamily · **BAFF-R:** BAFF receptor · **BCMA:** B cell maturation antigen · **FO:** follicular · **LTA:** lipoteichoic acid · **MZ:** marginal zone · **ODN:** oligodeoxynucleotide · **PGN:** peptidoglycan · **TACI:** transmembrane activator, calcium modulator, and cyclophilin ligand interactor · **TLR-A:** TLR agonist

Introduction

The B lymphocyte-activating factor belonging to TNF superfamily (BAFF; also called BlyS, THANK, TALL-1 or zTNF4) and a proliferation-inducing ligand (APRIL) are two cytokines expressed by myeloid cells [1] that act on B lymphocytes through two shared receptors: the transmembrane activator, calcium modulator, and cyclophilin ligand interactor (TACI) and the B cell maturation antigen (BCMA) [1]. A third receptor, called BAFF receptor (BAFF-R), selectively engages BAFF [1].

BAFF has been shown to be crucial in maintaining B lymphocyte homeostasis by facilitating the transition of B lymphocytes from T1 to T2 stage during the

maturation process. The pivotal role of BAFF in B lymphocyte survival and maturation has been demonstrated *in vivo* using a soluble BCMA molecule [2], and in genetically engineered mice that do not express BAFF, which have decreased levels of mature B cells and severely impaired antibody response against T-dependent and T-independent antigens [3, 4]. In contrast, transgenic mice expressing high levels of BAFF were shown to have increased numbers of circulating mature B cells [5–7], and develop a lupus-like autoimmune phenotype, which has been attributed to polyclonal activation of B cells. The role of APRIL is somewhat less clear although APRIL binding to TACI may play a role in T cell independent Ig isotype switching [8, 9].

Studies conducted in BAFF-R-deficient mice point to BAFF-R as the principal receptor transducing BAFF-mediated B lymphocyte survival signal [2]. TACI-deficient mice, on the other hand, have a selective deficiency in producing antibodies against T-independent type II antigens [10] and in IgA isotype switching [11]. Furthermore, it has been suggested that TACI may be balancing the B lymphocyte survival stimulus mediated by BAFF-R [12]. Finally, BCMA is shown to promote plasma cell survival [13] as well as induce antigen presentation by B cells [14].

TLR are crucial elements of the innate immune system that sense conserved pathogen-associated molecular patterns. Microbial TLR agonists (TLR-A) and their synthetic analogs typically induce the expression of co-stimulatory molecules and the secretion of cytokines by innate immune cells. TLR link the innate and adaptive immune system and lead to amplified immune response against microbial pathogens by activating T and B lymphocytes [15]. For example, it has been shown that the TLR9 agonist, CpG oligodeoxynucleotide (ODN), promotes isotype switching to IgG [16, 17]. Activation of B cells and induction of IgG secretion by CpG ODN, on the other hand, requires cytokines (IL-10, IL-15, IL-2) and/or a third signal such as anti-Ig, CD40L and BAFF [17–22]. Recently Ng and colleagues [23] have shown that CpG ODN up-regulate the expression of TACI and BAFF-R on B-1 and B-2 cells and amplifies BAFF-induced B cell survival. The effect of other TLR-A on TACI, BAFF-R and BCMA expression, and the effect of increased receptor expression on BAFF- or APRIL-mediated Ig secretion has not been studied.

This study shows that, in addition to CpG ODN [23], the TLR4 agonist LPS is able to up-regulate TACI expression while agonists for TLR2, TLR3 and TLR7 do not modulate the expression of BAFF/APRIL receptors. Furthermore, we show that the CpG ODN-induced up-regulation of TACI on follicular (FO) and marginal zone (MZ) B lymphocytes results in increased Ig secretion from the two B lymphocyte populations in response to BAFF or APRIL stimulation. These data suggest that

certain pathogen-associated molecular patterns sensitize B lymphocytes to BAFF- or APRIL-mediated Ig secretion by up-regulating TACI expression.

Results

The effect of TLR-A on the expression of TACI, BCMA and BAFF-R

In order to determine whether TLR-A affected the expression of the BAFF receptors, purified splenic B lymphocytes (purity more than 96%) were stimulated with R-837, ssRNA, poly(I:C), CpG ODN 1555, control ODN, LPS, peptidoglycan (PGN) or lipoteichoic acid (LTA) for 24 h and then the expression of TACI, BAFF-R and BCMA assessed by flow cytometry. As shown in Fig. 1A, C, cells stimulated with CpG ODN 1555 demonstrated a significant increase in TACI ($p < 0.05$) and BAFF-R expression ($p < 0.05$), but no significant change in BCMA expression. Cells stimulated with LPS showed increased TACI expression without a significant change in BAFF-R or BCMA expression (Fig. 1A). In contrast, cells stimulated with R837, ssRNA, poly(I:C), control CpG ODN, PGN, LTA, IFN- γ and IL-4 failed to significantly increase the expression of any of the BAFF/APRIL receptors.

To verify the flow cytometry results obtained with CpG ODN, mRNA levels of TACI, BAFF-R and BCMA were also analyzed on purified splenic B cells that were stimulated for 24 h with CpG ODN or control ODN. As shown in Fig. 1B, CpG ODN 1555 up-regulated mRNA expression of TACI and BAFF-R on B cells.

C and K, but not D type CpG ODN, up-regulate TACI and BAFF-R expression

Three different classes of synthetic CpG ODN have been described that have distinct structural and functional characteristics. The D type (also called A type [24]) CpG ODN are strong inducers of IFN- α from plasmacytoid DC [25]. The K type (also called B type [24]) CpG ODN are weak IFN- α inducers but they induce polyclonal B lymphocyte activation and proliferation as well as plasmacytoid DC maturation [26]. Recently, a third type of CpG ODN, called type C, which induces IFN- α production as well as polyclonal B cell activation, was described [27]. The CpG ODN 1555, which we used for the initial screening, corresponds to the K (B) type ODN class.

To determine whether the different types of CpG ODN had a differential effect on TACI, BCMA and BAFF-R expression, purified B cells were stimulated with K, D or C type ODN, and TACI, BCMA or BAFF-R levels were measured by flow cytometry. As shown in Fig. 2A,

the B and C type CpG ODN yielded similar increases in receptor expression, strongly up-regulating TACI expression and yielding a modest increase in BAFF-R expression. In contrast, D type ODN had no effect on any of the receptors' expression. None of the CpG ODN induced a change in BCMA expression (data not shown).

CpG ODN up-regulate TACI and BAFF-R expression through TLR9

CpG ODN are known to trigger cell signaling *via* TLR-9; however, recent studies suggested that CpG ODN might also induce TLR9-independent cell activation [28]. In order to establish whether the CpG ODN effect on TACI and BAFF-R expression was mediated through TLR9, we stimulated purified B lymphocytes from TLR9-KO mice and the wild-type C57BL/6 mice with CpG ODN 1555, CpG ODN 1612 or LPS, and measured receptor levels by flow cytometry. Unlike the C57BL/6 mice (data not shown), no increase in TACI or BAFF-R was observed in B cells of TLR9-KO mice (Fig. 2B). BCMA levels remained unchanged (data not shown). As expected, the absence of TLR9 did not modify the up-regulatory effect of LPS on TACI expression.

CpG ODN is a more potent inducer of BAFF-R expression than B cell mitogens

To determine the relative strength of CpG ODN-induced TACI and BAFF-R expression, we compared B lymphocyte TACI, BCMA and BAFF-R levels after stimulation with anti-IgM (10 μ g/mL), soluble CD40L (500 ng/mL), or CpG ODN (1 μ g/mL). Spleen cells were stimulated overnight and receptor expression levels were determined by flow cytometry on B220-gated cells. CpG ODN-induced up-regulation of TACI was markedly greater than that induced by the optimal concentration of soluble CD40L or anti-IgM antibodies (Fig. 3). Of note, higher anti-IgM (20 μ g/mL) or CD40L (2 μ g/mL) concentrations did not increase the up-regulation of TACI expression (data not shown). Neither CD40L nor anti-IgM antibodies increased the expression of BAFF-R or BCMA (data not shown). These results showed that CpG ODN-induced TACI and BAFF-R expressions are higher than those achieved using other B lymphocyte-mitogenic signals.

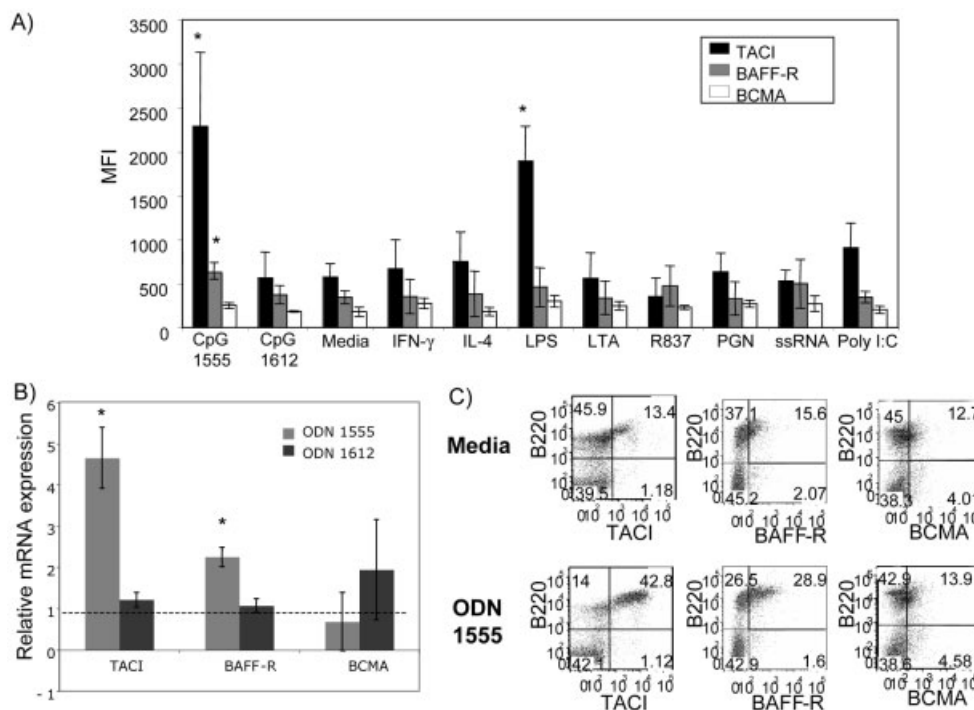


Figure 1. BALB/c mouse B lymphocyte TACI, BAFF-R and BCMA expression following TLR-A stimulation. (A) Purified B cells were incubated with TLR-A, IFN- γ or IL-4 for 24 h and MFI values of TACI, BAFF-R and BCMA were determined after flow cytometry assay. Changes in MFI values were compared to MFI values of medium-treated cells for statistical evaluation; * p <0.05 compared to medium-treated sample. (B) Purified B cells were incubated with CpG ODN 1555, CPG ODN 1612 or medium for 24 h and TACI, BCMA or BAFF-R mRNA levels were determined in real-time RT-PCR. Data are presented as relative levels to the medium-incubated sample, which is given an arbitrary value of 1 (dotted line). Average values of three to four independent experiments are plotted; * p <0.05 compared to medium-treated sample. (C) A representative dot blot showing B220⁺ and TACI⁺, BAFF-R⁺ or BCMA⁺ cells, following incubation of splenocytes with medium or CpG ODN 1555 for 24 h.

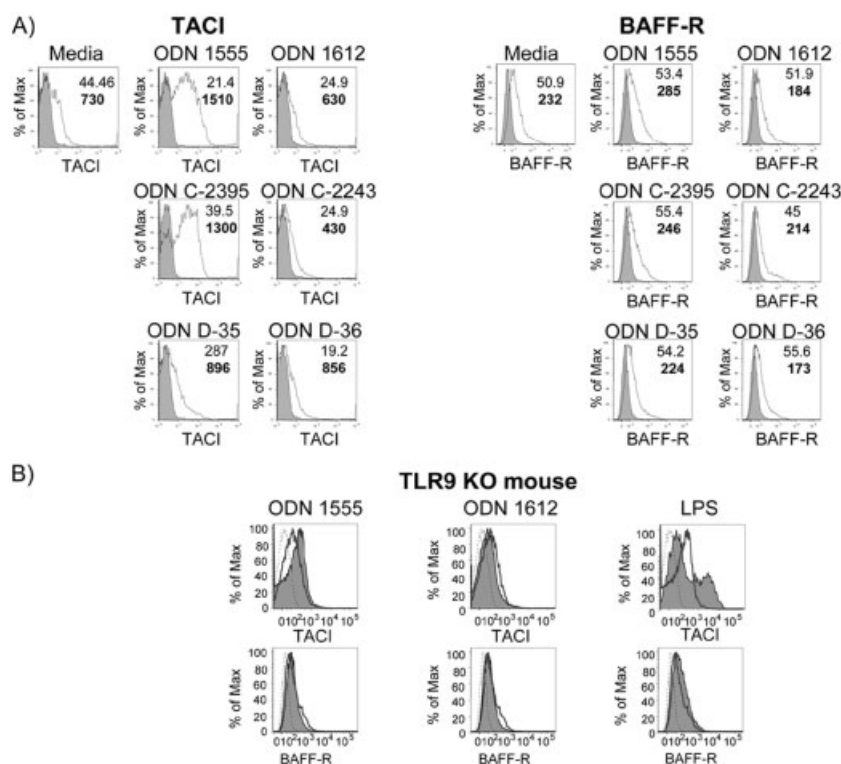
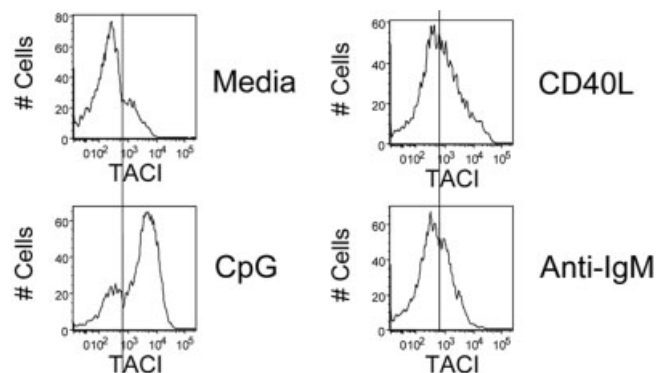


Figure 2. Role of TLR9 and the activity of different CpG ODN types in TACI and BAFF-R expression. (A) The effect of D (or A), K (or B) and C type CpG ODN on TACI expression was analyzed following incubation of BALB/c mice B lymphocytes with ODN 1555 (K type), ODN D-35 (D type), ODN C-2395 (C type), and medium. CpG ODN 1612, D-36 and C-2243 served as controls for K, D and C, respectively. Each histogram includes control isotype antibody (tinted) and CpG ODN-treated cells (straight line). Experiments were repeated four times with similar results. Histogram boxes include MFI values for the isotype antibody (upper panel) and antibody against TACI or BAFF-R (lower panel, bold). (B) The TLR9-KO mice TACI and BAFF-R levels were analyzed in FACS following treatment of B lymphocytes with CpG ODN 1555, CpG ODN 1612 (control), LPS or medium alone for 24 h. Each histogram includes control isotype antibody (thin), medium-treated cells (bold), and CpG ODN- or LPS-treated cells (tinted). Experiments were repeated two to three times and one of three similar results is presented.

CpG-ODN sensitizes B lymphocytes to BAFF- or APRIL-mediated immunoglobulin secretion

In order to understand whether the increased receptor expression makes B lymphocytes more responsive to BAFF and APRIL, the levels of Ig isotypes were determined in culture supernatants of B lymphocytes stimulated with BAFF or APRIL. B lymphocytes were pretreated with CpG ODN 1555 or medium for 24 h and then restimulated with BAFF, APRIL, LPS + IL-4, LPS + TGF- β , or left in medium alone for 7 days. Measurement of Ig levels in supernatants showed that CpG ODN 1555-pretreated B lymphocytes secreted significantly higher ($p < 0.05$) concentrations of Ig of all isotypes tested as compared to unstimulated B cells (Fig. 4). The increase in Ig production specifically suggests that CpG ODN pretreatment renders B lymphocytes more sensitive to BAFF and APRIL but not to stimuli that do not engage receptors for BAFF/APRIL, because there was no difference in the culture supernatant Ig levels of B lymphocytes that had been



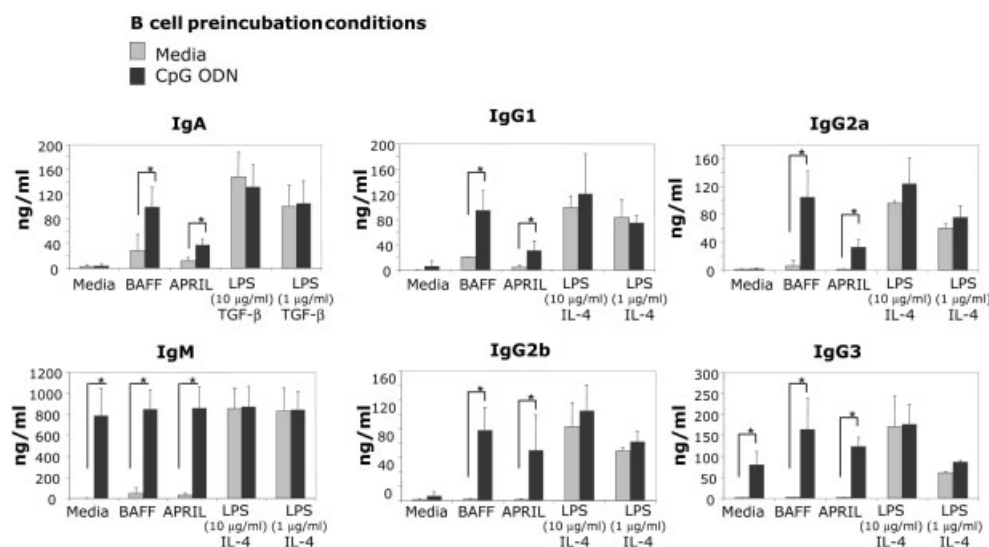


Figure 4. The effect of CpG ODN pretreatment on BAFF- and APRIL-induced Ig secretion. Purified BALB/c mouse B cells were initially incubated with the stimulatory CpG ODN 1555 (dark colored bars) or with medium (light colored bars) for 24 h, after which cells were washed once with complete RPMI medium, counted, and 2×10^6 cells from each group restimulated for 7 days with BAFF, APRIL, LPS + IL-4, LPS + TGF- β , or medium alone. Culture supernatant Ig concentrations were measured in an isotype ELISA. Mean Ig concentrations and standard deviations from three or four experiments were plotted; * $p < 0.05$ after comparison of Ig concentrations measured in culture supernatants of CpG ODN- and medium-only-pretreated B lymphocytes.

pretreated with CpG ODN or medium and restimulated with LPS + IL-4 or LPS + TGF- β .

Two different concentrations of LPS were used together with IL-4 or TGF- β to ensure that the absence of a difference in Ig concentration between CpG ODN- or medium-pretreated B cell culture supernatants was not a result of excessive LPS concentration (10 μ g/mL) leading to exhaustion of B cell Ig secretion capacity. Indeed, CpG ODN- or medium-pretreated cells secreted similar levels of Ig regardless of the LPS concentration used. The fact that there was a significant difference ($p < 0.05$) in IgA and IgG isotype concentrations between cell culture supernatants stimulated with 10 and 1 μ g/mL of LPS suggests that, at least at 1 μ g/mL concentration, LPS signal was not strong enough to exhaust the Ig secretion potential of B lymphocytes in medium-pretreated cells. It should be noted that the IgM levels in CpG ODN-pretreated/ BAFF- or APRIL-restimulated cells measured significantly higher than in medium-treated B lymphocytes; however, the levels did not exceed those induced by CpG ODN pretreatment alone. CpG ODN also induced significantly increased levels of IgG3 secretion ($p < 0.05$) compared to unstimulated B cells. Nevertheless, BAFF and APRIL further enhanced IgG3 secretion following CpG ODN pretreatment.

TACI is essential for increased immunoglobulin secretion induced by BAFF or APRIL

To assess the contribution of CpG ODN-induced TACI expression on the increased Ig secretion, CpG ODN-

pretreated B lymphocytes were incubated with BAFF or APRIL in the presence of increasing concentrations of antibodies against TACI or the control goat Ig for 7 days and the culture supernatants were tested for Ig levels. Interestingly, anti-TACI antibodies (5 μ g/mL) almost completely blocked CpG ODN/BAFF- (Fig. 5A) and CpG ODN/APRIL- (Fig. 5B) induced IgA and IgG production. The reduction in Ig levels was concentration-dependent and the control Ig did not inhibit Ig induction at the concentrations shown.

In order to rule out the possibility of an anti-TACI antibody-mediated negative signaling in B cells, we determined B lymphocyte survival and analyzed B lymphocyte responses to mitogens that do not use TACI as a receptor. Two additional mitogenic signals were used to stimulate B lymphocytes in the presence of anti-TACI antibodies. The highest concentration of anti-TACI antibodies (5 μ g/mL) that were inhibitory for BAFF or APRIL did not inhibit CD40L + IL-4-, LPS + TGF- β - or LPS + IL-4-induced Ig secretion from B cells (Fig. 6). LPS was used at two concentrations (1000 and 300 ng/mL) to rule out the possibility of a strong LPS stimulus that may overcome the possible negative signaling through TACI. The effect of neutralizing antibodies to TACI on B lymphocyte survival was investigated by incubating medium- or CpG ODN-pretreated B lymphocytes with anti-TACI or control antibodies (5 μ g/mL). Determination of viable cell percentages with the Via-Probe™ kit after 7 days of incubation revealed that there was no difference in the percentage of viable cells between the cells that had

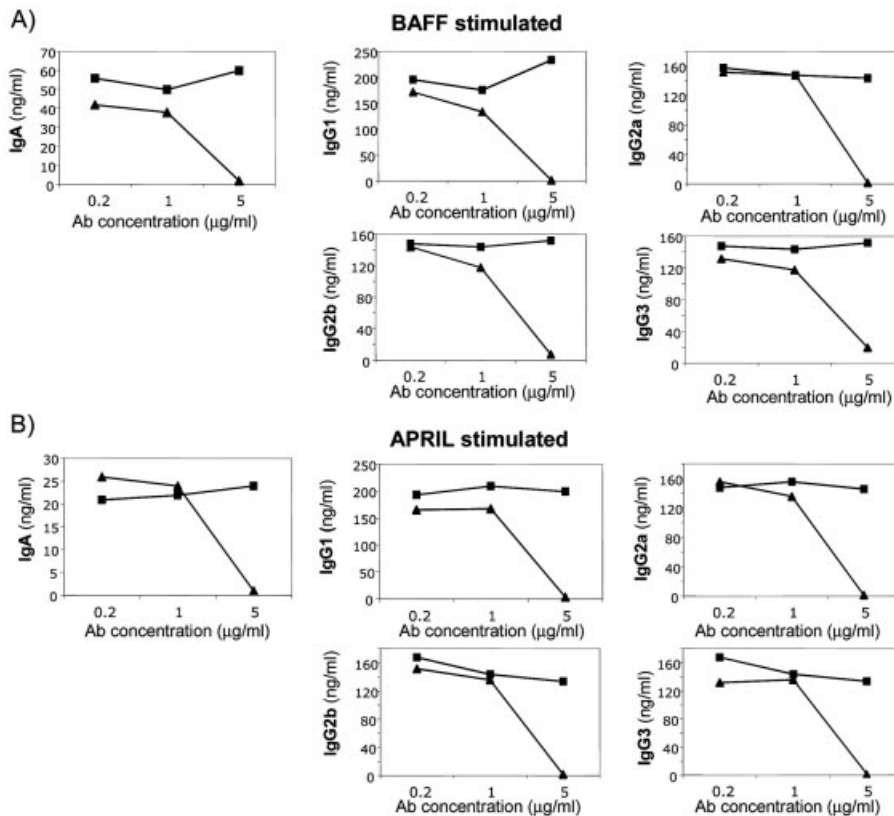


Figure 5. Inhibition of BAFF- and APRIL-induced Ig secretion by antibody against TACI. Purified BALB/c mouse B cells were initially incubated with the stimulatory CpG ODN 1555 for 24 h. Subsequently, cells were washed once with complete RPMI medium, counted and seeded at $2 \times 10^6/\text{mL}$. Cells were restimulated with BAFF (A) or APRIL (B) in the presence of increasing concentrations of polyclonal goat antibody against TACI (triangles) or control goat Ig (squares) for 7 days. Culture supernatant Ig concentrations were measured in an isotype ELISA. One of two experiments with similar results is shown.

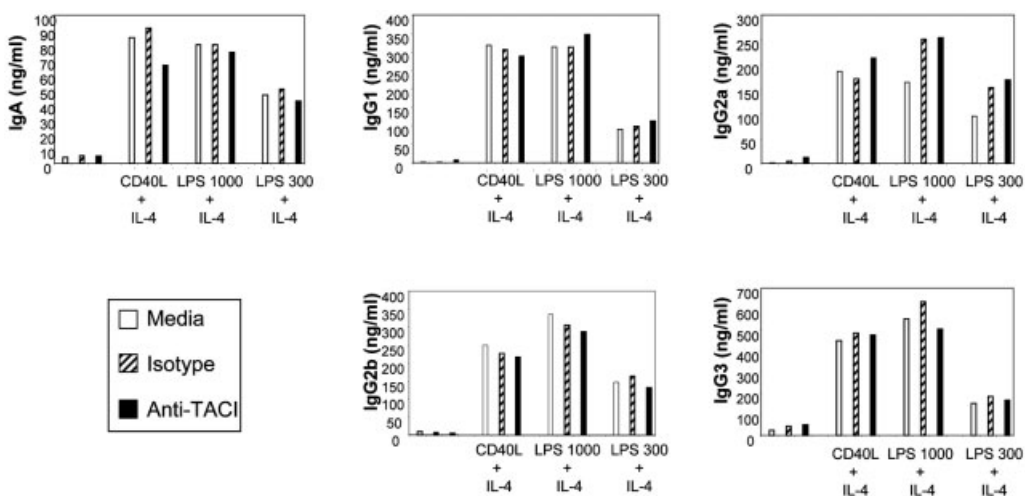


Figure 6. The effect of anti-TACI antibodies on CD40L- or LPS-mediated Ig secretion. Purified BALB/c mouse B cells were initially incubated with the stimulatory CpG ODN 1555 for 24 h. Subsequently, cells were restimulated for 7 days with CD40L + IL-4, LPS + IL-4, or LPS + TGF- β . Each stimulus included anti-TACI antibodies (5 $\mu\text{g}/\text{mL}$) (Anti-TACI), the control goat Ig (5 $\mu\text{g}/\text{mL}$) (Isotype) or medium only (Media) conditions. LPS was added at 1000 ng/mL or 300 ng/mL concentrations. Culture supernatant Ig levels are plotted. One of two experiments with similar results is shown.

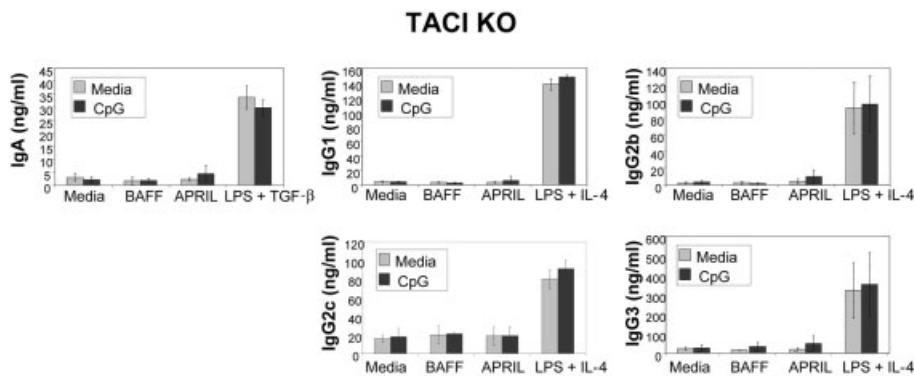


Figure 7. The effect of CpG ODN pretreatment on BAFF- or APRIL-mediated Ig secretion in TACI-KO mice. Purified B cells from TACI-KO mice or the control C57BL/6 mice (data not shown) were initially incubated with the stimulatory CpG ODN 1555 (dark colored bars) or with medium (light colored bars) for 24 h, after which cells were washed once with complete RPMI medium, counted, and 2×10^6 cells from each group restimulated for 7 days with BAFF, APRIL, LPS + IL-4, LPS + TGF- β , or medium alone. Culture supernatant Ig concentrations were measured in an isotype ELISA. Mean Ig concentrations and standard deviations from three experiments were plotted; * $p < 0.05$ after comparison of Ig concentrations measured in culture supernatants of CpG ODN- and medium-only-pretreated B lymphocytes.

been incubated with or without antibodies (Supporting Information Fig. 1).

Nevertheless, to definitively determine the role of TACI in CpG ODN-mediated augmentation of Ig secretion in BAFF- or APRIL-stimulated B lymphocytes, we used TACI-KO mice. As shown previously, TACI-KO mice B lymphocytes did not secrete Ig in response to BAFF or APRIL stimulation [29] (Fig. 7). Moreover, similar to results obtained with anti-TACI blocking antibodies, CpG ODN pretreatment did not render TACI-

KO mice B lymphocytes responsive to BAFF- or APRIL-mediated stimulation of Ig.

CpG ODN up-regulates TACI on FO and MZ B lymphocytes

Having determined that CpG ODN stimulates TACI expression on total splenic B lymphocytes, and CpG ODN-mediated TACI up-regulation plays a role in increased Ig secretion in response to BAFF or APRIL

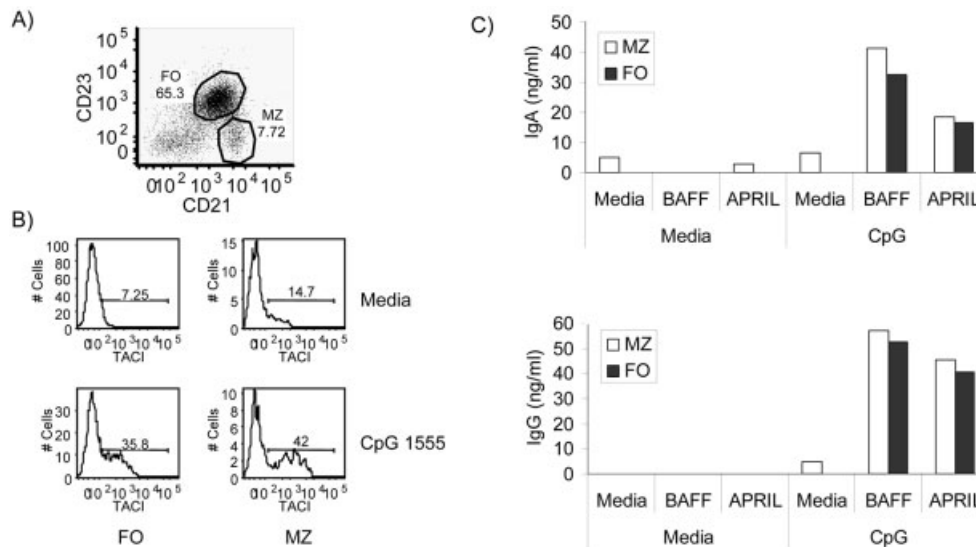


Figure 8. CpG ODN-induced up-regulation of TACI on FO and MZ B lymphocytes and their response to BAFF and APRIL. (A) A representative dot blot showing the gating of FO and MZ B lymphocytes on purified B lymphocytes is shown. FO and MZ B lymphocyte percentages are indicated on dot blot. (B) BALB/c mouse spleen cells were incubated with CpG ODN or medium alone for 24 h and B220⁺ cells were used to determine TACI expression on gated FO and MZ B lymphocytes. Numbers indicate the percentages of TACI⁺ cells in the gated area. One of three experiments with similar results is shown. (C) Sorted FO and MZ B lymphocytes were preincubated with medium or CpG ODN for 24 h and equal numbers of CpG ODN- or medium-pretreated FO or MZ B lymphocytes were stimulated with BAFF or APRIL. Culture supernatant IgA and IgG concentrations were determined in ELISA after 7 days of incubation. One of two experiments with similar results is shown.

stimulation, we next investigated the role of TACI expression on FO or MZ B lymphocytes stimulated with CpG ODN. As previously reported [30], TACI expression on MZ (CD21^{hi} CD23^{lo}) cells was higher than that on B220-gated FO (CD21⁺ CD23⁺) cells (Fig. 8A). However, following CpG ODN stimulation, TACI expression was up-regulated on both FO and MZ B lymphocytes. Further, prestimulation of sorted FO and MZ B lymphocytes with CpG ODN for 24 h, and restimulation with BAFF or APRIL for 7 days, led to a comparable increase in IgA and IgG secretion from both B lymphocyte populations (Fig. 8B). These results confirm the role of TACI in the CpG ODN-induced increase in BAFF- and APRIL-mediated IgA and IgG secretion.

Discussion

In this study we demonstrate that among the TLR-A tested, only TLR4 and TLR9 agonists up-regulated the TACI, while CpG ODN also increased BAFF-R expression on B cells. As the studies on CpG ODN-pretreated B lymphocytes demonstrated, CpG ODN primes B lymphocytes in a TLR9-dependent manner to up-regulate the expression of TACI, resulting in increased Ig output in response to BAFF or APRIL.

Previous studies had shown that BAFF plays a key role in B lymphocyte maturation by promoting the transition of cells from T1 to T2 stage and plasma cell survival. Together with APRIL, BAFF facilitates Ig isotype switching and augments Ig secretion [1]. Of the three BAFF receptors, BAFF-R is critical in transducing B lymphocyte survival signals [31] and in the development of antibody response against T-dependent antigens [31, 32]. TACI, on the other hand, is important in the generation of antibody response against T-independent type II antigens [10, 31]. TACI and BAFF-R are also implicated in T lymphocyte-independent Ig isotype switching, as BAFF and APRIL induce isotype switching to IgA through TACI [9, 11, 29], and BAFF promotes IgG1 and IgE isotype switching through BAFF-R [29].

Our studies showed that CpG ODN with a phosphorothioate backbone (types K and C), and to a lesser degree LPS, up-regulated the expression of TACI. A significant increase in BAFF-R expression was only induced by CpG ODN 1555. The effect of CpG ODN on B-1 and B-2 lymphocyte TACI and BAFF-R expression has recently been demonstrated by Ng and colleagues [23]. In a proliferation assay, they have shown that CpG ODN-induced BAFF-R expression led to an increase in BAFF-mediated B-1 and B-2 lymphocyte response.

Here we determined that, in addition to rendering B lymphocytes sensitive to BAFF-induced proliferation,

the ligation of TLR9 also sensitizes splenic B lymphocytes (B-2 cells) to BAFF- or APRIL-mediated IgA and IgG secretion. This response was specific for BAFF and APRIL, as the Ig output in response to control stimuli, LPS + IL-4, or LPS + TGF- β was not increased in CpG ODN-pretreated B lymphocytes. It should be noted that the increase in all Ig isotypes as a result of BAFF or APRIL stimulation indicates that in this experimental system, not only naive B lymphocytes' response was measured but also those that have already undergone isotype switching. Indeed, it has been shown that CpG ODN promotes isotype switch to IgG1, IgG2 and IgG3 in humans [17], and IgG2a, IgG2b and IgG3 in mice [16].

Although both TACI and BAFF-R expression is increased with CpG ODN, the fact that APRIL stimulation of CpG ODN-pretreated B lymphocytes leads to increased Ig secretion suggested that the TACI up-regulation is responsible for the APRIL-mediated effect, because APRIL binds to TACI and BCMA but not to BAFF-R. Unlike APRIL, BAFF binds to BAFF-R and TACI; therefore both of these receptors may be mediating BAFF-induced Ig secretion. In order to understand whether the increased TACI expression is responsible for BAFF- or APRIL-mediated stimulation of Ig secretion, we tested the effect of CpG ODN on B cells in the absence of TACI engagement to BAFF or APRIL. Inclusion of a polyclonal antibody against TACI completely ablated the IgG and IgA secretion in CpG ODN-pretreated and BAFF- or APRIL-stimulated B lymphocytes. These results were confirmed in experiments where B lymphocytes from TACI-KO mice were used. As shown previously [29], B lymphocytes from TACI-KO mice did not secrete Ig in response to BAFF or APRIL stimulation. Moreover, CpG ODN pretreatment did not render TACI-KO mice B lymphocytes responsive to BAFF or APRIL stimulation.

Interestingly, this differs from the report of He and colleagues [17] which showed that IL-10 was required for CpG ODN- and BAFF-mediated stimulation of IgG production. Several differences between our experimental system and theirs may account for the disagreement. Firstly, in our experiments we preincubated B lymphocytes with CpG ODN for 24 h prior to stimulation with BAFF or APRIL, while He and colleagues simultaneously stimulated B lymphocytes with CpG ODN and BAFF. The preincubation step in our experiments may have provided sufficient time for the up-regulation of TACI expression. Secondly, differences in B lymphocyte responses may be species-specific since we used mouse B lymphocytes while He and colleagues investigated human B lymphocyte responses. Finally, the potency of the CpG ODN used in two studies may be different because the sequences of the CpG ODN used in two studies are not identical.

It is now well established that TACI is the key BAFF/APRIL receptor in transducing signals for IgA isotype switch in mouse and in humans. Mice deficient in TACI [10] and individuals with homozygous mutations in the *TNFRSF13B* gene, encoding TACI [29], manifested defective isotype switching to IgA. Although there is no report on CpG ODN-mediated isotype switching to IgA, studies have shown that CpG ODN-containing vaccines induce IgA antibodies [33] and CpG ODN-treated mononuclear cells secrete IgA [34]. Our findings suggest that CpG ODN-induced TACI up-regulation may be responsible for the increased IgG and IgA antibody response observed in vaccines containing CpG ODN [24, 33, 35].

Given the role of TACI in the generation of antibody response against T-independent type II antigens, the effect of CpG ODN on TACI expression may have interesting implications for bacterial polysaccharide vaccines. Bacterial polysaccharides are poor immunogenic antigens and the immune responses of infants to bacterial polysaccharides vaccines are especially compromised [36]. Together with B-1 cells, MZ B lymphocytes are the primary B lymphocytes that are responsible for the development of antibody response against bacterial polysaccharide vaccines [37]. Other studies have shown that TACI is preferentially expressed on MZ B lymphocytes [30]. The fact that TACI-KO mice respond poorly to bacterial polysaccharides reiterates the role of TACI-expressing MZ B lymphocytes in the development of antibody response against bacterial polysaccharide vaccines.

In this study, we determined that CpG ODN strongly up-regulates TACI expression on both FO and MZ B lymphocytes, and, when pretreated with CpG ODN, otherwise unresponsive FO and MZ B lymphocytes secrete IgA and IgG in response to BAFF or APRIL stimulation. Based on these observations, it is reasonable to assume that increased TACI expression may be playing a role in the generation of antibody response in vaccines composed of T-independent type II antigens and CpG ODN [35, 38].

We have shown that CpG ODN strongly up-regulated TACI expression on FO and MZ B lymphocytes while the effect on BAFF-R expression was modest. Increased expression of TACI rendered B lymphocytes more susceptible to BAFF and APRIL stimulation. Since TLR-A are considered as vaccine adjuvants, understanding their effects on molecules that regulate the B cell arm of the adaptive immune system may lead to selection of TLR-A as adjuvants for vaccines that seek amplified humoral immune response and also help to better evaluate vaccines containing TLR-A as adjuvants. Moreover, unraveling the increased responsiveness of B lymphocytes to BAFF and APRIL as a result of up-regulated TACI expression may help to understand the

pathogenesis of autoimmune diseases where TLR9-mediated polyclonal activation of B lymphocytes is responsible for autoreactive antibody development.

Materials and methods

Antibodies and reagents

Antibodies to TACI-PE, BCMA-FITC, BAFF-R and isotype controls were obtained from R&D Systems, Inc. (Minneapolis, MN) as were IFN- γ , IL-4, APRIL, purified goat anti-TACI antibody, purified control goat Ig and TGF- β . Rat IgG2a-PE-Cy5, anti-rat IgG-FITC, rat IgG2b-PE, anti-CD21-FITC, anti-CD21-PE, anti-CD23-biotin, streptavidin-allophycocyanin-Cy7 and B220-PE-Cy5 antibodies were purchased from BD Biosciences Pharmingen (San Jose, CA). Rat IgG2a-Alexa Fluor and anti-B220-Alexa Fluor antibodies were purchased from Caltag (Burlingame, CA). Cell viability detection kit (BD Via-Probe™) was also from BD Biosciences Pharmingen. Poly(I:C), staphylococcal PGN, R-837 (Imiquimod) and ssRNA were purchased from Invivogen (San Diego, CA). LTA and *Escherichia coli* LPS were from Sigma-Aldrich (St. Louis, MO). Soluble mouse CD40L and the cross-linking enhancer were bought from Alexis Corporation (Lausen, Switzerland), while anti-IgM antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA).

CpG ODN were synthesized at the Center for Biologics Evaluation and Research core facility. The ODN and their sequences used in this study were ODN 1555, GCTAGACGT-TAGCGT; ODN 1612, GCTAGAGCTTAGGCT; ODN C-2395, TCGTTCGTTTTCGGCGCGCGCCG; ODN C-2243, GGGGGAG-CATGCTGGGGGGG; ODN D-35, GGTGCATCGATG-CAGGGGGG; and ODN D-36, GTGGCATCTATGCAGGGGGG (underlined nucleotides show the position of CpG motifs). Ig isotype ELISA kit was purchased from Sigma-Aldrich and Ig isotype standards were from BD Biosciences Pharmingen. Determination of culture supernatant IgG2c concentrations was done using a capture antibody from Southern Biotechnology (Birmingham, AL) and a reference serum from Bethyl Laboratories, Inc. (Montgomery, TX). ELISA development reagents were from KPL (Gaithersburg, MD).

Animals

Female mice, 6–12 wk old, were used in all experiments. BALB/c and C57BL/6 mice were from The Jackson Laboratory (Bar Harbor, ME). TACI-KO mice were a kind gift of Dr. Richard J. Bram (Mayo Clinic, Rochester, MN). TLR9-KO mice, which were generated by Shizuo Akira (Osaka University) on a C57BL/6 background, were a kind gift of Dr. Dennis Klinman (FDA, CBER, Bethesda, MD).

Determination of TACI, BCMA and BAFF-R expression

The expression of BALB/c mouse B cell TACI, BAFF-R and BCMA was assessed by flow cytometry and real-time RT-PCR. C57BL/6, TACI-KO and TLR9-KO mice TACI, BAFF-R, and BCMA expressions were also analyzed using flow cytometry. B cells were first purified from splenocytes by negative

selection using magnetic cell separation kit (Miltenyi Biotec, Inc., Auburn, CA) as described by the manufacturer, and 1×10^6 cells were seeded in a 48-well plate for stimulation. Purified B cells were suspended in RPMI medium supplemented with 10% fetal bovine serum and with penicillin-streptomycin (complete RPMI), and stimulated with CpG ODN (1 $\mu\text{g/mL}$), poly(I:C) (100 $\mu\text{g/mL}$), PGN (5 $\mu\text{g/mL}$), LTA (10 $\mu\text{g/mL}$), LPS (10 $\mu\text{g/mL}$), R-837 (5 $\mu\text{g/mL}$), ssRNA (5 $\mu\text{g/mL}$), IFN- γ (10 ng/mL) or IL-4 (5 ng/mL) for 24 h. After 24 h of incubation, receptors were measured in flow cytometry using antibodies against TACI, BCMA or BAFF-R. Average receptor MFI values were calculated using FlowJo Software (Tree Star Corporation, Ashland, OR). TACI, BAFF-R and BCMA expressions were also analyzed in BALB/c mice spleen cells that were stimulated for 24 h with anti-IgM, CD40L and enhancer. Antibodies against B220 were used to detect and gate B lymphocytes.

B lymphocyte TACI, BCMA and BAFF-R mRNA expressions were determined by RT-PCR after the treatment of purified B lymphocytes for 24 h with CpG ODN 1555 or 1612. Total RNA was extracted using RNeasy Mini kit (Qiagen, Valencia, CA) and 300–1000 ng/sample of RNA was reverse-transcribed into cDNA using First-strand cDNA Synthesis Kit (Amersham Biosciences, Little Chalfont, UK) as per manufacturer's instructions. Relative mRNA levels for receptor genes were analyzed using the corresponding TaqMan Gene Expression assay by real-time PCR (Applied Biosystems, Foster City, CA). Values for each target gene were normalized using human 18S RNA. Expression values were calculated using the $2^{-\Delta\Delta C_t}$ method [39] and expressed relative to the unstimulated sample. TACI, BAFF-R and BCMA expression levels were also measured following the stimulation of spleen cells with different concentrations of anti-IgM or CD40L plus the enhancer.

Stimulation of B cells and measurement of immunoglobulin isotypes

The effect of CpG ODN-induced increase in TACI and BAFF-R expression on BAFF- or APRIL-induced Ig secretion was tested in an *in vitro* B lymphocyte culture system. Purified BALB/c, TACI-KO or C57BL/6 mice B lymphocytes or purified FO and MZ B lymphocytes were seeded at 2×10^6 – 4×10^6 cells/2 mL and stimulated with CpG ODN (1 $\mu\text{g/mL}$) or medium for 24 h. Stimulated B lymphocytes were washed and seeded (2×10^6 /mL/well) into 48-well plates for stimulation with medium alone, BAFF (1 $\mu\text{g/mL}$), APRIL (1 $\mu\text{g/mL}$), LPS (10 $\mu\text{g/mL}$ or 1 $\mu\text{g/mL}$) with IL-4 (5 ng/mL), or LPS (10 $\mu\text{g/mL}$ or 1 $\mu\text{g/mL}$) with TGF- β (50 ng/mL). For stimulation of FO and MZ B lymphocytes, 5×10^5 cells were incubated in 96-well plates in 500 μL of medium and stimulated with BAFF (1 $\mu\text{g/mL}$), APRIL (1 $\mu\text{g/mL}$) or medium alone.

After 7 days of stimulation, culture supernatants were collected and Ig isotype concentrations were measured by ELISA. Because TACI-KO mice are on C57BL/6 background, and C57BL/6 mice have IgG2c rather than IgG2a isotype, we measured IgG2c in the culture supernatants of B lymphocytes from C57BL/6 and TACI-KO mice. Ig isotype standards were used to quantify the response.

In other experiments, increasing concentrations of purified polyclonal antibodies against TACI were included in CpG ODN-pretreated BALB/c mice B cell cultures together with BAFF (1 $\mu\text{g/mL}$), APRIL (1 $\mu\text{g/mL}$), CD40L (2 $\mu\text{g/mL}$) and enhancer (1 $\mu\text{g/mL}$) + IL-4, LPS (1000 or 300 ng/mL) + IL-4, or LPS (1000 or 300 ng/mL) + TGF- β (5 ng/mL). Similar concentrations of purified goat Ig were used as a control for anti-TACI antibodies.

Detection of TACI on FO and MZ B lymphocytes and cell sorting

The expression of TACI on FO and MZ B lymphocytes was assessed on BALB/c mice splenocytes. Cells were stained with fluorescent-conjugated B220, CD21, CD23 and TACI antibodies. FO (CD21⁺ CD23⁺) and MZ (CD21^{hi} CD23^{lo}) B lymphocytes were identified on B220-gated B lymphocytes in flow cytometry assay and TACI expression was determined on FO- or MZ-gated cells.

The response of CpG ODN-pretreated FO or MZ B lymphocytes to BAFF and APRIL stimulation was tested after sorting of FO and MZ B lymphocytes. For sorting of FO and MZ B lymphocytes, purified B lymphocytes obtained after collagenase/DNase I digestion were stained with a mixture of CD21-PE and CD23-FITC antibodies. Cells were washed, resuspended in PBS supplemented with 5 mM EDTA, 1% FCS, and sorted on the FACSARIA instrument using FACSDiVa software (BD Biosciences). Sorted FO and MZ B lymphocytes (2.5×10^6 /mL to 5×10^6 /mL) were stimulated with CpG ODN (1 $\mu\text{g/mL}$) or left in medium for 24 h. The next day, following two washing steps, 2×10^5 cells were reseeded in 200 μL of medium for stimulation with BAFF (1 $\mu\text{g/mL}$), APRIL (1 $\mu\text{g/mL}$) or medium alone for 7 days. Culture supernatant IgA and total IgG concentrations were measured using a sandwich ELISA composed of capture antibody and a peroxidase-conjugated detection antibody.

Determination of B lymphocytes viability

To determine cell survival, B lymphocytes from BALB/c mice were stimulated with CpG ODN or medium alone for 24 h. The next day, cells were harvested, washed and counted. Equal numbers of cells were cultured in the presence or absence of goat anti-TACI antibody or purified goat Ig (control antibody) for 7 days. Cell viability was determined by staining with Via-Probe™ (7-amino-actinomycin D) and analyzed by flow cytometry according to the manufacturer's (BD Biosciences Pharmingen) instructions. Percent viable cells were expressed by gating 7-amino-actinomycin D-negative cell population.

Statistical analysis

Student's *t*-test was used for statistical analysis.

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