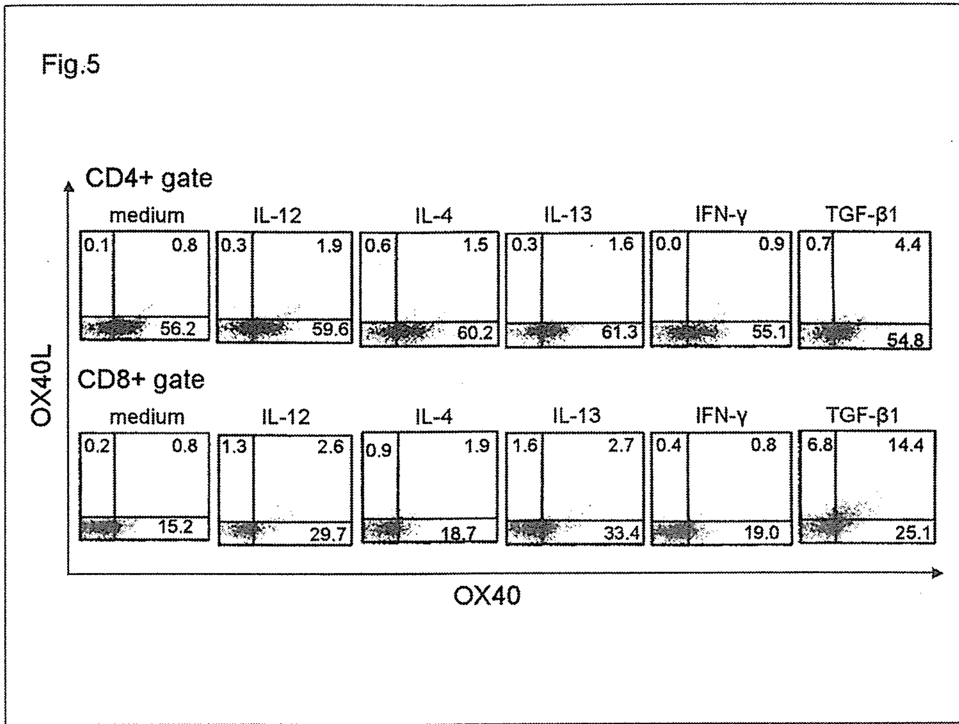

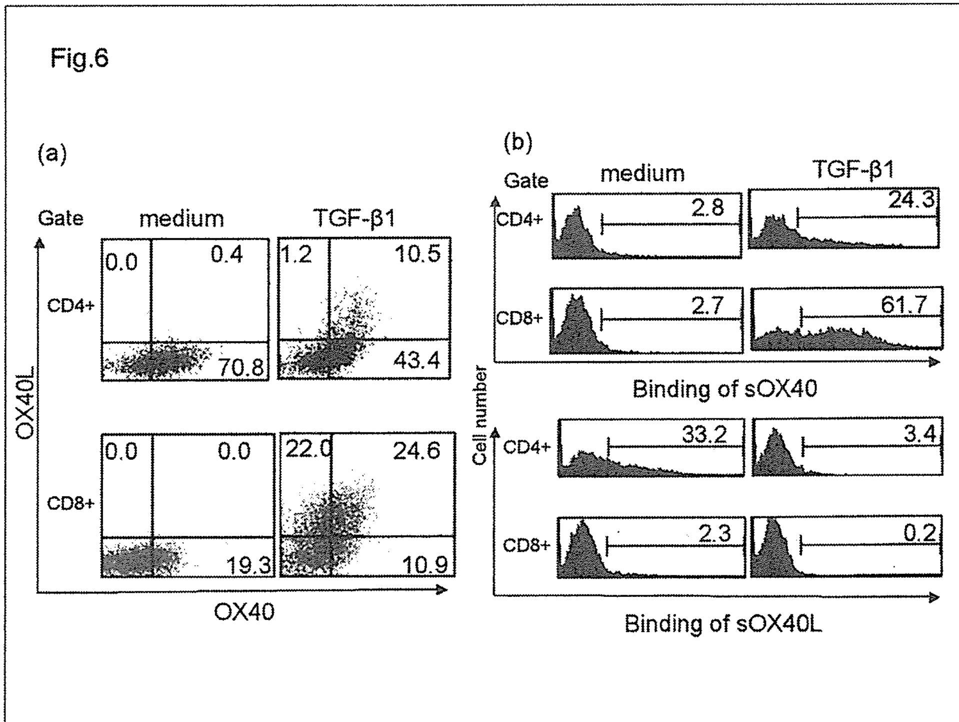

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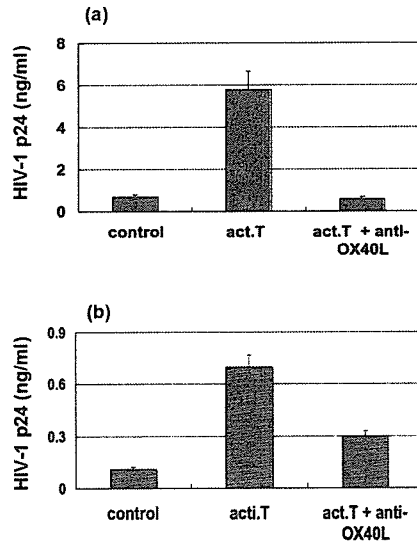
Fig.7



TACI attenuates antibody production costimulated by BAFF-R and CD40

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B cell activating factor of the TNF family (BAFF), plays critical roles in B cell survival, activation, differentiation, and antibody (Ab) production. BAFF binds to three receptors: BAFF-R, transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) and B cell maturation antigen. While BAFF-R is the primary receptor for B cell costimulation by BAFF, TACI is reported to serve as a positive or negative regulator for B cell responses depending on conditions. To determine the real role of TACI in B cell responses, we examined the functional relationship between TACI and BAFF-R in Ab production from human peripheral blood B cells using agonistic mAb. BAFF-R and CD40 enhanced IgG secretion and B cell proliferation, which were inhibited by TACI. Although TACI induced mild B cell apoptosis, its extent did not correlate with that of TACI-mediated inhibition of IgG secretion. In addition, TACI inhibited B-lymphocyte-induced maturation protein-1 expression, IgG secretion from previously IgG-negative selected B cells, and activation-induced cytidine deaminase expression enhanced by BAFF-R and CD40. Importantly, BAFF-R and CD40 enhanced B cell responsiveness to TACI-mediated suppression. Thus, BAFF may attenuate T cell-independent and -dependent B cell responses by TACI.

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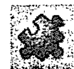
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Introduction

B cell activating factor of the TNF family (BAFF, also known as BLyS, TALL-1, THANK, zTNF4, TNFSF13b, and CD257) is a potent B cell survival and maturation factor, which costimulates B cell responses such as Ab production and class switching [1]. Mice overexpressing BAFF display mature B cell hyperplasia and symptoms of systemic lupus erythematosus and Sjögren's syndrome [2–4]. Conversely, BAFF-deficient mice have significantly fewer marginal zone and follicular B cells [5, 6]. In humans, aberrant expression of BAFF was reported in patients with autoimmune diseases [2, 7–10] and B cell malignancies [11, 12].

BAFF binds to three receptors belonging to the TNF receptor family: B cell maturation antigen (BCMA) (CD269), transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) (CD267) and BAFF receptor (BAFF-R), also called BR3 (CD268) [1]. Knockouts of BAFF-R but not TACI or BCMA produced a BAFF-deficient phenotype in mice, indicating that

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Abbreviations: AID: activation-induced cytidine deaminase · **BAFF:** B cell activating factor of the TNF family · **BCMA:** B cell maturation antigen · **Blimp-1:** B-lymphocyte-induced maturation protein-1 · **CVID:** common variable immunodeficiency · **PB-B cells:** peripheral blood B cells · **TACI:** transmembrane activator and calcium-modulator and cyclophilin ligand interactor

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BAFF/BAFF-R interactions are primarily responsible for B cell survival and responses [1, 13–16]. In contrast, genetic studies in mice have demonstrated the inhibitory role of TACI in B cell survival, proliferation, and CD40-mediated Ab production and that TACI could directly induce apoptosis in certain conditions [17, 18]. However, it is also reported that TACI-deficient mice reveal reduced IgA production and compromised humoral responses to T-independent Ag [19] and that defects in TACI are associated with two forms of human immunodeficiency, common variable immunodeficiency (CVID) and IgA deficiency [20, 21], suggesting the facilitating role of TACI in B cell responses.

While BAFF- and CD40L (CD154)-induced B cell responses are well characterized, the role of TACI and its relationship to BAFF-R and CD40 costimulatory functions in B cell responses are poorly understood, especially in humans. By examining the *in vitro* responses in human peripheral blood B (PB-B) cells using agonistic anti-BAFF-R and anti-TACI mAb, we found that in addition to the induction of B cell apoptosis, TACI signaling altered B cell proliferation and Ab production such as B lymphocyte-induced maturation protein-1 (Blimp-1) expression, IgG secretion from previously IgG-negative selected B cells, and activation-induced cytidine deaminase (AID) expression enhanced by BAFF-R and CD40 signaling. Importantly, BAFF-R and CD40 signaling rendered B cells susceptible to TACI-mediated suppression. Thus, BAFF may negatively regulate through TACI not only T cell-independent B cell responses by BAFF-R, but also T cell-dependent B cell responses by CD40.

Results

TACI suppresses Ab production by BAFF-R and CD40

To clarify TACI functions in B cell responses, we first compared the effects of agonistic mAb to BAFF-R (8A7) or TACI (11H3) on Ab production from human PB-B cells *in vitro*. IgG secretion was enhanced by BAFF, CD40L and anti-BAFF-R mAb in the presence of IL-2, which was more evident after BCR ligation. However, anti-TACI mAb inhibited IgG secretion augmented by anti-BAFF-R mAb and CD40L although anti-TACI mAb alone showed no significant enhancement or inhibition of IgG secretion (Fig. 1A). Consistent with this, B cell proliferation was also enhanced by BAFF, CD40L and anti-BAFF-R mAb, which was inhibited by anti-TACI mAb (Fig. 1B). It should be noted that anti-BAFF-R mAb was always effective in enhancing IgG secretion and B cell proliferation more than BAFF in different doses tested and that anti-BAFF-R mAb and anti-TACI mAb exhibited enough agonistic effects without their immobilization on a solid phase (data not shown). These results suggest that TACI suppresses Ab production enhanced by BAFF-R and CD40.

TACI inhibits BAFF-R- and CD40-enhanced B cell survival

It has been reported that TACI can directly induce apoptosis under certain conditions [17]. To determine the contribution of TACI-mediated apoptosis to the

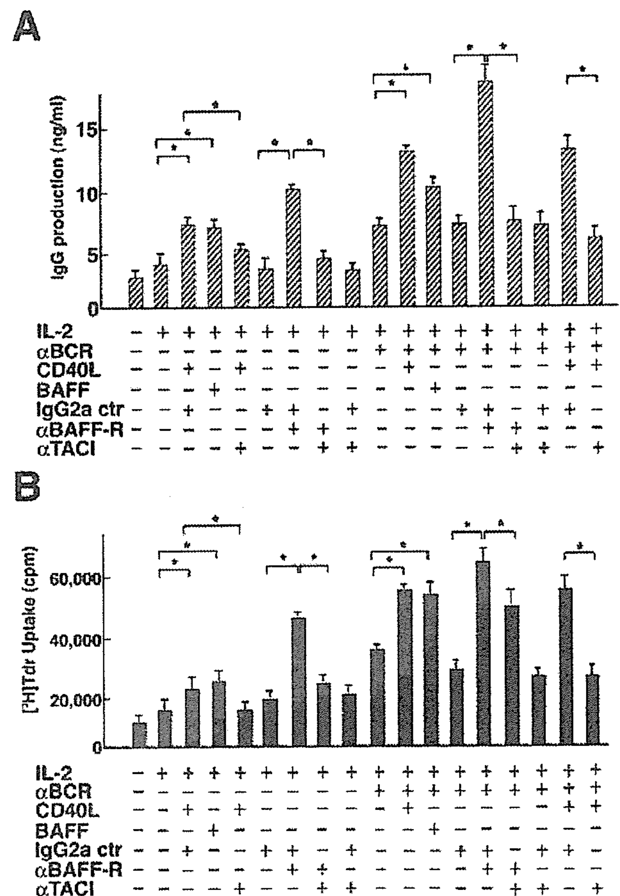

Figure 1. Effects of anti-TACI mAb on IgG secretion and B cell proliferation enhanced by anti-BAFF-R mAb and CD40L. Human PB-B cells (1×10^5/well) were cultured in triplicate in 96-well plates with BAFF (1 μg/mL), CD40L (2 μg/mL), anti-BAFF-R mAb (8A7, 5 μg/mL), isotype-matched control mAb (5 μg/mL) plus or minus anti-Igκ and anti-Igλ Ab (1 μg/mL) together with or without anti-TACI mAb (11H3, 10 μg/mL) in the presence of IL-2 (25 U/mL). (A) IgG concentration of supernatant was measured by ELISA after 10-day culture. (B) [^{3}H]Thymidine [0.5 μCi (18.5 kBq)/well] incorporation of B cells was measured during the last 18 h of 72-h culture. Anti-Igκ and anti-Igλ Ab were used to mimic Ag stimulation and sufficient secretion of Ig *in vitro*. IL-2 was used for B cell proliferation, differentiation and Ig secretion *in vitro*. Data are means ± SD and are representative of three independent experiments. * $p < 0.05$.

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suppression of BAFF-R- and CD40-enhanced Ab production, we examined the effect of anti-TACI mAb on apoptosis of human PB-B. Fig. 2A shows that B cell apoptosis was induced by anti-TACI mAb or anti-Fas mAb alone after 3-day culture. This TACI-mediated B cell apoptosis was slightly inhibited by BCR ligation, which was further inhibited by anti-BAFF-R mAb and CD40L. In addition, Fig. 2B shows that the number of viable B cells after culture correlated with IgG secretion and B cell proliferation observed in Fig. 1, suggesting that TACI inhibited B cell survival enhanced by BAFF-R and CD40. However, the inhibitory effect of anti-TACI mAb on IgG secretion per cell was still observed even after standardization by the number of survival cells (Fig. 2C). These results suggest that TACI-mediated suppression of BAFF-R- and CD40-enhanced Ab production cannot be accounted for solely by B cell apoptosis directly by TACI.

TACI inhibits plasma cell generation and IgG class switching

To clarify the mechanisms of inhibition other than apoptosis by TACI in Ab production, we examined the direct effects of anti-TACI mAb on the expression of Blimp-1 in human PB-B cells after BAFF-R and CD40 stimulation. Blimp-1 is a transcriptional repressor and drives terminal differentiation of B cells into Ig-secreting plasma cells [22, 23]. The expression of Blimp-1 up-regulated rapidly by anti-BAFF-R mAb and CD40L within 1 day was clearly inhibited by anti-TACI mAb (Fig. 3), suggesting that TACI may inhibit plasma cell generation. As it has been reported that BAFF can induce Ig class switching in human *in vitro* [24], we examined the effect of anti-TACI mAb on IgG secretion from previously IgG-negative selected human PB-B cells enhanced by BAFF-R and CD40. As shown in Fig. 4A, anti-BAFF-R mAb, BAFF and CD40L could induce substantial IgG secretion from previously IgG-negative selected B cells. However, anti-TACI mAb resulted in twofold decrease in IgG secretion by anti-BAFF-R mAb and CD40L, which was more evident after BCR ligation. Flow cytometric analysis showed that the number of IgG expressing B cells almost correlated with the extent of IgG secretion (data not shown). Because Ig class switching needs AID expression and activation, we then examined AID expression in PB-B cells incubated under the above conditions. AID was found to be up-regulated by BAFF, CD40L or anti-BAFF-R mAb, which was further enhanced by BCR ligation (Fig. 4B). As expected, this enhanced AID expression was significantly inhibited by anti-TACI mAb, which would result in the inhibition of IgG secretion from previously IgG-negative selected PB-B cells. These results suggest that TACI-mediated suppression of BAFF-R- and CD40-enhanced Ab produc-

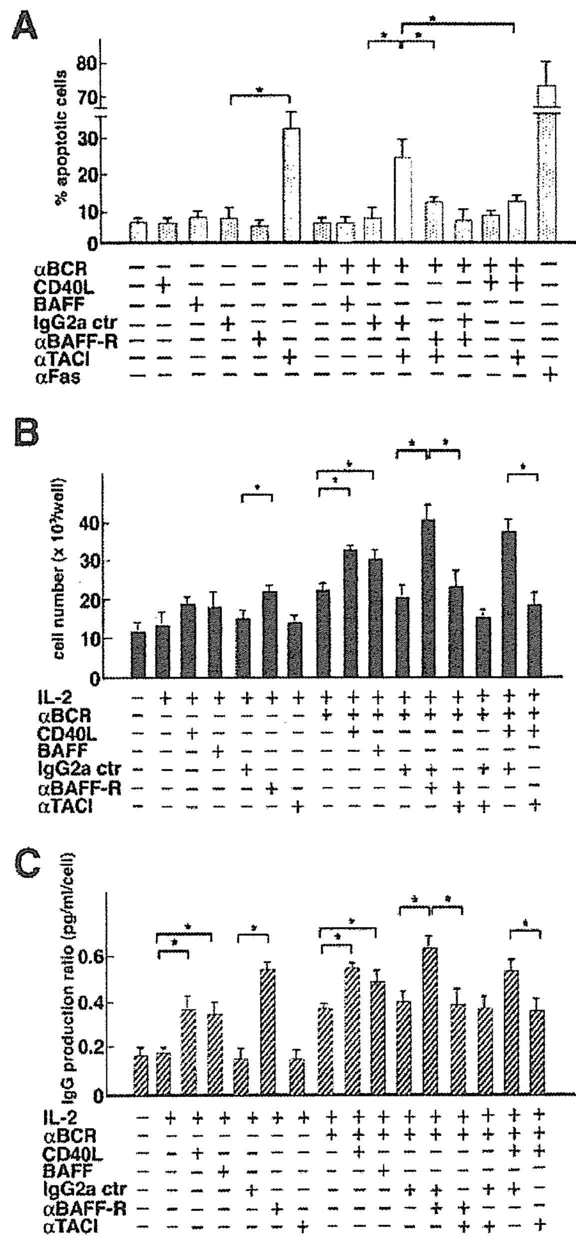

Figure 2. Effects of anti-TACI mAb on B cell apoptosis, cell survival and IgG secretion per cell enhanced by anti-BAFF-R mAb and CD40L. (A) Human PB-B cells (4×10^5/ well) were treated with BAFF (1 μg/mL), CD40L (2 μg/mL), anti-BAFF-R mAb (8A7, 5 μg/mL), isotype-matched control mAb (5 μg/mL), anti-Fas mAb (0.5 μg/mL), with or without anti-TACI mAb (11H3, 10 μg/mL) in the presence or absence of anti-Igκ and anti-Igλ Ab (1 μg/mL) for 3 days. The proportion of cells positive for annexin V was measured by flow cytometry. (B) Under the conditions specified in Fig. 1A, viable cell number (per well) was determined by the trypan blue exclusion methods. (C) Under the conditions specified in Fig. 1A, the amount of IgG secretion was standardized by viable B cell numbers (B). Anti-Igκ and anti-Igλ Abs were used to mimic Ag stimulation and sufficient secretion of Ig *in vitro*. IL-2 was used for B cell proliferation, differentiation and Ig secretion *in vitro*. Data are means ± SD and are representative of three independent experiments. *$p<0.05$.

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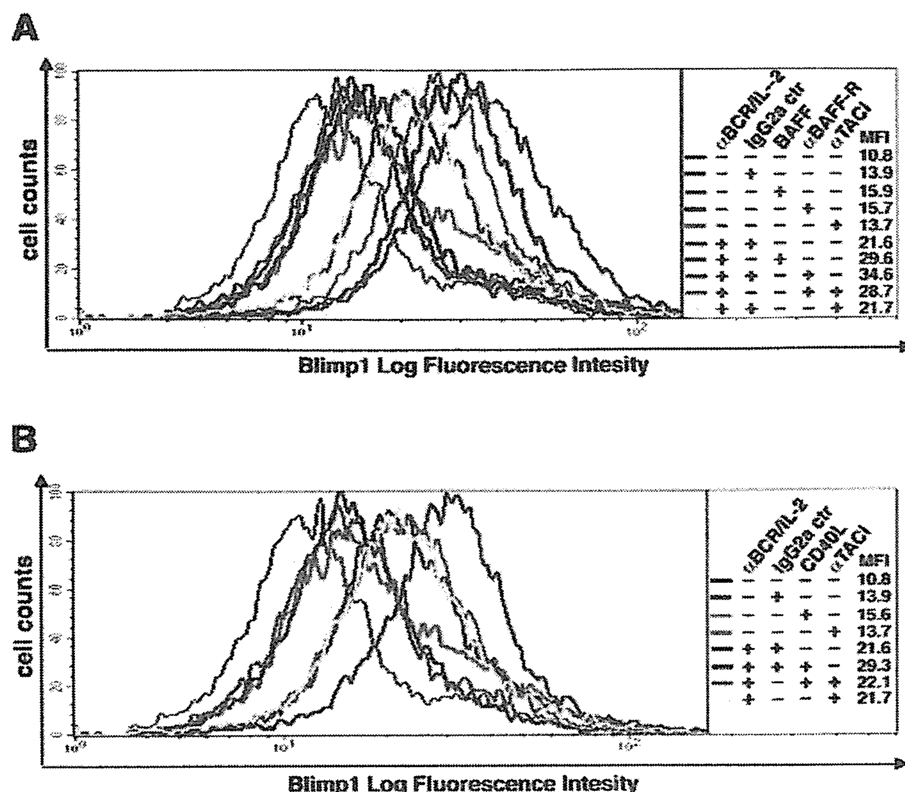

Figure 3. Effects of anti-TACI mAb on Blimp-1 expression enhanced by anti-BAFF-R mAb and CD40L. Human PB-B cells (1×10^5/well) were treated with anti-BAFF-R mAb (8A7, 5 μg/mL) (A), CD40L (2 μg/mL) (B), isotype-matched control mAb (5 μg/mL) together with or without anti-TACI mAb (11H3, 10 μg/mL) in the presence or absence of anti-Igκ and anti-Igλ Abs (1 μg/mL) for 1 day. The expression level of Blimp-1 was measured by flow cytometry. Anti-Igκ and anti-Igλ Abs were used to mimic Ag stimulation and IL-2 was used for B cell proliferation, differentiation to plasma cells *in vitro*. Data are representative of three independent experiments.

tion may result from inhibited plasma cell generation and IgG class switching by the reduced expression of Blimp-1 and AID.

BAFF-R and CD40 enhance B cell responsiveness to TACI-mediated suppression

Finally, to understand how BAFF may act through TACI to inhibit BAFF-R- and CD40-enhanced Ab production, we examined the effects of anti-BAFF-R mAb and CD40L on TACI expression on human PB-B cells. Anti-BAFF-R mAb and CD40L up-regulated TACI expression for at least 4 days (Fig. 5A). As expected, PB-B cells treated with anti-BAFF-R mAb or CD40L for 3 days showed a significantly lower IgG secretion and B cell proliferation in response to BAFF and anti-TACI mAb but not to CD40L, compared to PB-B cells treated with isotype-matched control mAb (Fig. 5B and C). These results strongly suggest that BAFF costimulates B cells through BAFF-R initially, and then terminates sustained and/or excess B cell responses by BAFF-R and CD40 by up-regulating TACI expression.

Discussion

In the present study, we demonstrated that TACI-specific signaling inhibited both BAFF-R- and CD40-enhanced Ab production from human peripheral blood B cells *in vitro*, although TACI-specific signaling directly induced mild B cell apoptosis. These results suggest that BAFF may regulate both T cell-independent and -dependent B cell responses through a dynamic balance between TACI and BAFF-R/CD40 signaling.

The precise molecular mechanisms of TACI inhibition of Ab production enhanced by BAFF-R and CD40 (Fig. 1A) are unknown. This inhibitory effect of TACI was not due to down-regulation of BAFF-R or CD40 because the levels of BAFF-R and CD40 surface expression on B cells were unchanged after TACI ligation (data not shown). However, it appears that TACI mediates signals for inhibiting Blimp-1 up-regulation and plasma cell generation, inhibiting AID induction and Ig class switching, and inducing B cell apoptosis (Figs. 2–4). Both BAFF-R and CD40 deliver a signal for B cell survival and maturation, induction of Ab

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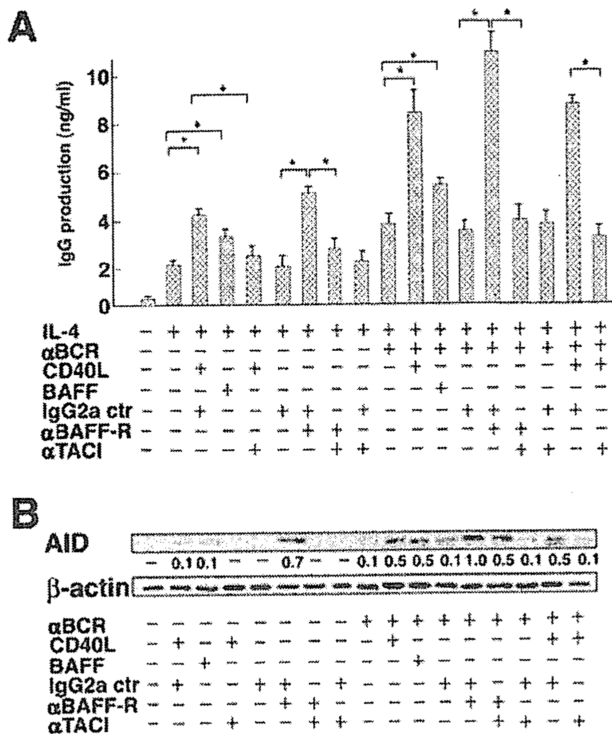

Figure 4. Effects of anti-TACI mAb on IgG secretion from previously IgG-negative selected B cells and AID expression enhanced by anti-BAFF-R mAb and CD40L. Human IgG-negative PB-B cells (1 × 10^5/ well) were cultured in triplicate in 96-well plates with BAFF (1 μg/mL), CD40L (2 μg/mL), anti-BAFF-R mAb (8A7, 5 μg/mL), isotype-matched control mAb (5 μg/mL) plus or minus anti-Igκ and anti-Igλ Ab (1 μg/mL) together with or without anti-TACI mAb (11H3, 10 μg/mL) in the presence of IL-4 (20 U/mL). (A) IgG concentration of supernatant was measured by ELISA after 10-day culture. (B) After culture for 3 days, cell lysates were prepared and subjected to immunoblot analysis using anti-AID Ab or anti-β-actin Ab (β-actin; loading control). The levels for AID were analyzed by a LumiVision analyzer and presented as relative fold of the maximum level. Anti-Igκ and anti-Igλ Ab were used to mimic Ag stimulation and sufficient secretion of Ig *in vitro*. IL-4 was used for B cell proliferation, differentiation, and IgG class switching and secretion *in vitro*. Data are means ± SD and are representative of three independent experiments. *p <0.05.

production and Ig class switching, based on activation of non-canonical NF-κB pathway [1, 25]. Thus, it is considered that BAFF-R- and CD40-mediated non-canonical NF-κB pathway plays an important role in B cell responses and is controlled by the same or similar mechanisms, although this is not clarified yet. The common adaptor molecule for both receptors is TRAF3, and thus its behavior in B cells is speculated to be essential for the propagation of BAFF and CD40L signaling pathways. Thus, the relationship between TACI signaling and the non-canonical NF-κB pathway is currently being investigated in our laboratory.

As reported previously [17], we also showed that TACI signaling directly induced mild B cell apoptosis (Fig. 2A). Moreover, the reduced B cell proliferation by TACI (Fig. 1B) would also weaken various B cell activities. Thus, B cell responses enhanced by BAFF and CD40L would consequently be terminated by TACI. It is possible that TACI inhibitory signals regulate the cell cycle [26].

Interestingly, we found enhanced IgG secretion from previously IgG-negative selected B cells and AID induction by CD40 and BAFF-R, and their suppression by TACI (Fig. 4B), indicating the negative role of TACI in Ig class switching. However, it has been reported in mice that both BAFF-R and TACI could mediate Ig class switching by BAFF and APRIL [27]. It was also reported that TACI-deficient mice exhibited deficient humoral responses to T-independent Ag [19]. Furthermore, two recent papers on patients with TACI-deficient CVID reported that the patients showed hypogammaglobulinemia including IgA deficiency [20, 21]. These findings indicate the positive role of TACI in B cell responses. It is possible that Ag stimulation in the absence of BAFF and CD40L signals renders B cells gradually responsive to TACI stimulation, because B cell responses were observed at later time points (Fig. 5) but not at early time points (Fig. 1). This might be relevant to impaired humoral responses to T-independent Ag in TACI-deficient mice. In patients with TACI-deficient CVID, however, one reported that BAFF but not APRIL induced IgG production *in vitro* [20] while the other reported that both BAFF and APRIL did not induce IgG production *in vitro* [21]. Thus, it is possible that TACI gene deficiency does not solely account for the observed phenotype of patients with TACI-deficient CVID and that yet unidentified additional genetic alterations are involved in the patients. On the other hand, it has been reported that APRIL-deficient mice have impaired IgA production [28]. In addition, recent reports revealed that APRIL interacts with heparan sulfate proteoglycans [29, 30]. Proteoglycans such as syndecans and CD44 have glycosaminoglycan chains and can signal for cellular responses [31]. Thus, it is quite possible that TACI engagement alone is insufficient and both TACI and glycosaminoglycan engagements are required for Ig class switching, because anti-TACI mAb alone could not induce both IgG secretion from previously IgG-negative selected B cells and AID expression (Fig. 4). This might be relevant to the positive role of TACI observed in the above B cell responses. However, we cannot rule out BCMA-TACI synergism in mediating Ab production and Ig class switching. The possibility is currently being investigated in our laboratory.

Although we discussed TACI-mediated inhibition of IgG class switching based on the results that TACI inhibited IgG secretion from previously IgG-negative

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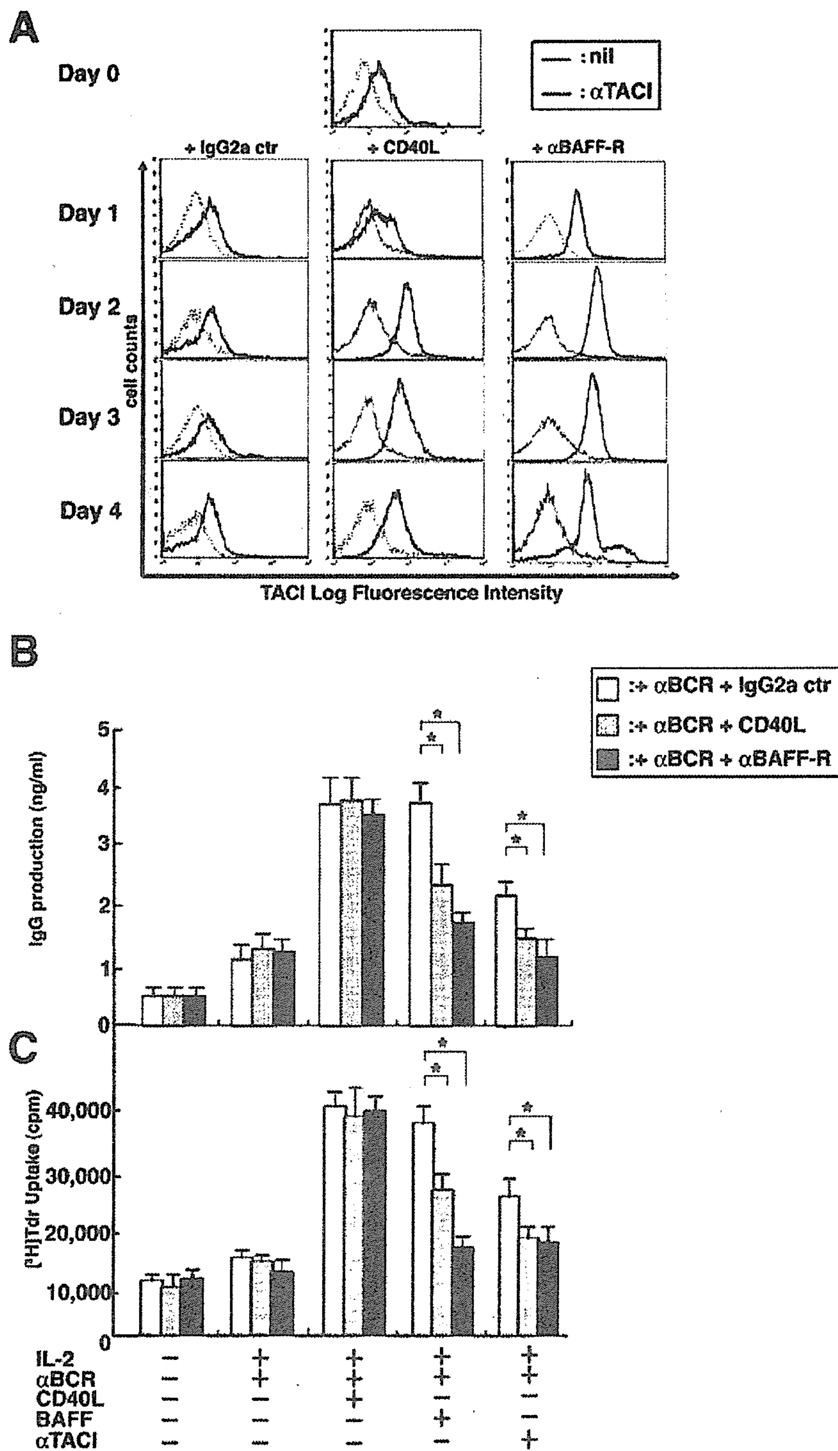

Figure 5. Effects of anti-BAFF-R mAb and CD40L on B cell responses to TACI. (A) Human PB-B cells (1×10^5/well) were treated with anti-BAFF-R mAb (8A7, 2.5 μg/mL), CD40L (2 μg/mL) or isotype-matched control mAb (5 μg/mL) in the presence of anti-Igκ and anti-Igλ Ab (1 μg/mL) for the indicated periods. The expression level of TACI was measured by flow cytometry with anti-TACI mAb (11H3). Dark lines: staining with anti-TACI mAb, light lines: isotype controls. (B, C) After cultured for 3 days under the above conditions, the cells (1×10^5/well) were cultured in triplicate in 96-well plates with BAFF (1 μg/mL), CD40L (2 μg/mL), anti-TACI mAb (11H3, 10 μg/mL) or isotype-matched control mAb (2.5 μg/mL) with anti-Igκ and anti-Igλ Ab (1 μg/mL) in the presence of IL-2 (25 U/mL). IgG concentration of supernatant was measured by ELISA after 10-day culture (B). [^{3}H]Thymidine [0.5 μCi (18.5 kBq)/well] incorporation of B cells was measured during the last 18 h of a total of 72-h culture (C). Anti-Igκ and anti-Igλ Ab were used to mimic Ag stimulation and sufficient secretion of Ig *in vitro*. IL-2 was used for B cell proliferation, differentiation and IgG secretion *in vitro*. Data shown are representative of three independent experiments. $^*p < 0.05$.

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selected B cells enhanced by BAFF-R and CD40, there are some arguments that our data reflect amplification of IgG synthesis by already switched B cells rather than *de novo* switching in naïve B cells because human IgG-negative PB-B cells are heterogeneous as they comprise both naive and memory B cells in contrast to those in mice [32]. Although ~25% of IgG^- memory B cells (*i.e.* IgM^+CD27^+ B cells) are present in human PB-B cells [32], these cells are the circulating form of splenic marginal zone B cells [33], which are shown to undergo Ig class switching [34]. In addition, we confirmed the correlation of the number of IgG-expressing B cells with the extent of IgG secretion and that TACI inhibited AID expression enhanced by BAFF-R and CD40 (Fig. 4B).

Finally, we showed that anti-BAFF-R mAb and CD40L stimulated B cells to up-regulate TACI expression and become susceptible to TACI-mediated suppression (Fig. 5). CD40- and BAFF-R-mediated TACI up-regulation was observed at a high level at least for 4 days, indicating that the inhibitory effects of TACI are not transient and are rather steadily involved in CD40- and BAFF-R-mediated B cell responses. These results may explain how BAFF kinetically transmits the positive and negative signals into B cells through BAFF-R and TACI. In the early stage of B cell activation, BAFF preferentially transmits the positive signal *via* BAFF-R, and costimulates B cells. Simultaneously, BAFF-R signaling up-regulates the cell surface TACI expression and its availability by out-competing BAFF-R for binding to BAFF, which may make BAFF costimulation shift to mediating the negative signal in the later stages of T cell-independent and -dependent B cell activation. This is quite reminiscent of the roles of CD28 and CTLA-4 in regulation of T cell activation [35]. We previously reported abundant expression of BAFF by follicular dendritic cells in germinal centers of human lymph nodes and a possibly sequential link between T cell-independent and -dependent B cell responses in the germinal centers by BAFF [36]. Thus, it is possible that up-regulation of TACI expression serves as a negative feedback against excess B cell activation mediated by BAFF-R and CD40 stimulation in the germinal centers. As APRIL shares TACI with BAFF [1], it is possible that APRIL can also deliver a negative signal for B cell responses by TACI.

In conclusion, our present study showed that BAFF could negatively regulate through TACI not only T cell-independent B cell responses by BAFF-R, but also T cell-dependent B cell responses by CD40. This new role of BAFF in regulating B cell responses in concert with CD40L suggests that humoral immune responses require an extremely complex system for amplification, fine tuning and termination. Analyzing the involvement of TACI dysfunction in the pathogenesis of autoimmune diseases and B cell malignancy will be helpful for its further understanding and is currently underway in our laboratory.

Materials and methods

Antibodies and reagents

The following mAb and polyclonal Ab were used in the present study: human BAFF-R (8A7) and human TACI (11H3) (eBioscience, San Diego, CA); AID (C-20) and Blimp-1 (N-20) (Santa Cruz Biotechnology, Santa Cruz, CA); β-actin (AC-40) (Sigma-Aldrich, St. Louis, MO); Fas (CH11) (Immunotech, Marseille, France); Igκ (G20–193) and Igλ (JDC-12) as B cell receptor (BCR) (BD PharMingen, San Diego, CA). The following reagents were used: recombinant human IL-2 (Invitrogen, San Diego, CA), recombinant human IL-4, recombinant human CD40L and recombinant human BAFF (PeproTec, Rocky Hill, NJ); control mouse IgG2a (UPC 10) and control goat IgG (Sigma-Aldrich).

Cell preparation and cell cultures

Human PBMC were isolated from healthy donors by centrifugation with Ficoll-Hypaque, and purified B (PB-B) cells were isolated by depletion of non-B cell populations using a B cell isolation kit and autoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). The resultant B cell population was <2% $CD14^+$, <1% $CD3^+$, <2% $CD57^+$, and >95% $CD20^+$. IgG^- PB-B cells were prepared with a B cell isolation kit, biotinylated anti-human IgG (G18–145, BD PharMingen) and autoMACS. The resultant IgG^- PB-B-cell population was <3% IgG^+. PB-B cells were cultured in RPMI 1640 medium supplemented with 25 mM HEPES, 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 5.5×10^{-2} mM β-mercaptoethanol, 100 U/mL penicillin, and 100 U/mL streptomycin (all from Invitrogen). PB-B cells consistently express BAFF-R, TACI, IgM, IgG, and CD40.

Flow cytometric analysis

After incubation of arbitrary Ab with 1–2 μg/10^6 PB-B cells for 20 min on ice, cells were washed and resuspended in propidium iodide solution, and analyzed using FACScalibur and associated Cell Quest software (both from Becton Dickinson, Mountain View, CA). For intracellular staining of Blimp-1, cells were fixed with PBS containing 1% formaldehyde, followed by 80% EtOH. Fixed cells were washed with saponin-PBS (PBS containing 0.1% saponin, 0.1% BSA, 0.1% NaN_3, and 0.01 M HEPES). After washing, cells were resuspended in saponin-PBS and stained with anti-Blimp-1 Ab, followed by washing with saponin-PBS. FITC-labeled goat Ab to mouse IgG2a (#M32201, Caltag, Burlingame, CA) or swine Ab to goat IgG (#G50001, Caltag) was used as a second Ab. Isotype-matched mouse IgG2a control (UPC 10, Sigma-Aldrich) or goat IgG control (Sigma-Aldrich) was used to evaluate the background.

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Immunoblot analysis

PBS-washed cell pellets (4×10^6 cells) were resuspended with 0.5% SDS solution and boiled for 5 min. Proteins (5–8 μg) were separated by SDS-PAGE, transferred to an Immobilon-P (Millipore, Bedford, MA) or Trans-Blot nitrocellulose (Bio-Rad, Hercules, CA) membrane, blocked with 5% skim milk, and immunoblotted with arbitrary Ab and horseradish peroxidase (HRP)-labeled secondary Ab (#NA931V and #NA934V; Amersham Biosciences, Arlington Heights, IL) using the immunoreaction enhancer solution (Can Get Signal; Toyobo, Osaka, Japan). Blotting was developed by using the enhanced chemiluminescent substrate (SuperSignal West Pico; Pierce, Rockford, IL) and visualized with a LumiVision analyzer (Taitec, Tokyo, Japan). The densitometric analysis was performed by using a LumiVision analyzer. All immunoblots were examined more than three times.

Detection of apoptotic cells

To detect apoptotic cells, phosphatidylserine exposure was measured by using annexin V-FITC apoptosis detection kit (BD PharMingen) and FACScalibur (Becton Dickinson) as described previously [37].

Proliferation assay

PB-B cells were cultured in a 96-well plate (1×10^5/well) with anti-BCR mAb (anti-Igκ and anti-Igλ, 1 μg/mL each) and BAFF or CD40L or anti-BAFF-R mAb and/or anti-TACI mAb in the presence or absence of IL-2 (25 U/mL). B cell proliferation was quantitated by pulsing the cells during the last 18 h of total 72 h culture with 0.5 μCi (18.5 kBq)/ well of [^{3}H]thymidine and measured in a liquid scintillation beta counter, TopCount NXT (Perkin Elmer-Cetus, Foster City, CA).

Detection and quantification of *in vitro* IgG secretion

PB-B or IgG-negative PB-B cells were cultured with anti-BCR mAb (anti-Igκ and anti-Igλ, 1 μg/mL each) and BAFF, CD40L, or anti-BAFF-R mAb and/or anti-TACI mAb in the presence or absence of IL-2 (25 U/mL) in a 96-well plate (1×10^5/well) for 10 days. To induce IgG class switching, we used IgG-negative PB-B cells and IL-4 (20 U/mL) instead of IL-2. IgG levels in the culture supernatants were determined by ELISA using anti-human Ig (#2010–01, Southern Biotechnology, Birmingham, AL) as the capture Ab and HRP-labeled goat anti-human IgG (#55252, ICN Biomedicals, Aurora, OH) as the detector Ab. After addition of p-nitrophenyl phosphate substrate (Sigma-Aldrich), the amount of IgG present was assessed by spectrophotometric analysis at 490 nm using a microplate reader (model 550, Bio-Rad) as described previously [38].

Statistical analysis

All data were expressed as mean ± SD. Differences between groups were examined for statistical significance using the paired *t*-test. A *p* value less than 0.05 denoted the presence of a statistically significant difference.

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TACI regulates IgA production by APRIL in collaboration with HSPG

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Transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) is a member of the tumor necrosis factor (TNF) receptor family that serves as a receptor for B-cell activating factor of the TNF family (BAFF) and as a proliferation-inducing ligand (APRIL). Although TACI is reported to function as a positive or negative regulator for B-cell responses, its roles remain elusive. Experiments using TACI siRNA into B cells indicated that TACI positively regulated APRIL-induced IgA production in collaboration with heparan sulfate proteoglycans (HSPG). Furthermore, TACI negatively regulated BAFF-induced B-cell proliferation and production of IgA and IgG. In addition, B cells treated with heparitinase to denature HSPG showed that HSPG is essential for APRIL-induced B-cell responses such as B-cell proliferation, IgG and IgA production, induction of activation-induced cytidine deaminase (AID), and noncanonical NF-κB2. In contrast, phosphorylation of physiological AID kinase, protein kinase A (PKA), was dependent on TACI. Importantly, coligation of TACI and HSPG by specific antibodies, but not by TACI or HSPG ligation itself, could induce the phosphorylation of PKA and IgA production instead of APRIL. Our findings indicate that simultaneous binding of TACI and HSPG on B cells with APRIL is crucial for IgA production. (Blood. 2007;109: 2961-2967)

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Introduction

Tumor necrosis factor (TNF) family ligands, a proliferation-inducing ligand (APRIL, CD256), and B-cell activating factor of TNF family (BAFF, also known as BLyS, TALL-1, THANK, zTNF4, TNFSF13b, and CD257) are implicated in several immunologic phenomena such as peripheral B-cell survival, CD154 (CD40L)–independent antibody isotype switching and production, autoimmunity, and tumor cell growth.[1,2] BAFF and APRIL bind to 2 receptors, BCMA (B-cell maturation antigen [TNFRSF 17 and CD269]) and TACI (transmembrane activator and calcium-modulator and cyclophilin ligand interactor [TNFRSF 13B and CD267]).[3-5] BAFF binds selectively to the third BAFF receptor, BAFF-R (TNFRSF 13C and CD268).[6,7] All these receptors are TNF receptor family molecules. On the other hand, APRIL interacts with heparan sulfate proteoglycans (HSPGs), which are structurally unrelated to TNF receptors and are likely a third receptor for APRIL.[8,9]

Studies on transgenic and knockout mice have indicated that BAFF/BAFF-R interactions are primarily responsible for B-cell survival and responses because the BAFF-deficient phenotype is characterized by a reduced number of splenic B cells and by insensitivity to T cell–dependent and T cell–independent antibody production[10] similar to that in BAFF-R–deficient mice.[11,12] In contrast, TACI-deficient mice show mature B-cell hyperplasia and autoimmunity, and TACI can directly induce apoptosis under certain conditions,[13-15] suggesting that TACI is a negative regulator of BAFF signaling in B-cell survival and responses. However, it is also reported that TACI-deficient mice exhibit reduced immunoglobulin A (IgA) production and compromised humoral responses to T-independent antigens (TI-Ags)[13] and that defects in TACI are associated with 2 forms of human immunodeficiency, common variable immunodeficiency (CVID) and IgA deficiency.[16,17] Thus, the real roles of TACI in B-cell responses remain obscure. On the other hand, studies on APRIL transgenic mice showed enhanced humoral responses to T-dependent (TD) and TI-Ags and a gradual increase in serum IgA level.[2,18] APRIL knockout mice have low serum IgA levels and impaired IgA responses, though conflicting results are reported.[2,19] In addition, in vitro studies demonstrated that APRIL enhances B-cell proliferation, plasmablast survival,[20] and class switch recombination (CSR) to IgG and IgA through the up-regulation of activation-induced cytidine deaminase (AID).[21]

Given the potential importance of TACI and APRIL in IgA production, we performed a series of experiments using a small interference RNA (siRNA) technique to knock down TACI and heparitinase treatment to denature HSPG on human peripheral blood B cells to assess the functional aspects of TACI and HSPG in B-cell responses. The studies reported here show the mutually close relationship between TACI and HSPG in APRIL-induced B-cell responses, especially in IgA production.

Materials and methods

Antibodies and reagents

The following antibodies were used: NF-κB1/p65, NF-κB2/p52, Lamin A, and AID (Santa Cruz Biotechnology, Santa Cruz, CA); polyclonal human TACI (Active Motif, Carlsbad, CA), PKA C-α, and phospho-PKA C (Thr197) (Cell Signaling Technology, Beverly, MA); β-actin and FLAG-M2 (Sigma-Aldrich, St Louis, MO); HSPG (10E4, mouse IgM, κ; Seikagaku, Tokyo, Japan); human BCMA (Alexis, Läufelfingen, Switzerland); human

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CD40 and CD19 (eBioscience, San Diego, CA); human IgA (Dako, Copenhagen, Denmark); human Igκ and Igλ, and mouse Igκ (BD PharMingen, San Diego, CA); and control mouse IgM and IgG2a (Sigma-Aldrich). The following reagents were used: heparitinase (MP Bioscience, Solon, OH); human IL-4, human soluble CD40L, human BAFF, and TGF-β (PeproTech, Rocky Hill, NJ); human APRIL-FLAG fusion protein (Mega-APRIL; Alexis); and 8-bromo-cAMP (Calbiochem, La Jolla, CA). Human BAFF-FLAG fusion protein was prepared as described previously.[22] Anti–human TACI mAb (11H3, mouse IgG2a,κ) was prepared by immunization with human TACI full-length cDNA-transfected cells. The specificity and agonistic activity of anti–human TACI mAb are demonstrated in Figure S1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Cell preparation and cell cultures

Human peripheral blood mononuclear cells were isolated from buffy coats by using centrifugation with Ficoll-Hypaque, and purified B cells were isolated by depletion of non–B-cell populations using a B-cell isolation kit and auto-MACS (Miltenyi Biotec, Bergisch Gladbach, Germany). The resultant B-cell population was less than 2% CD14$^+$, less than 1% CD3$^+$, less than 2% CD57$^+$, less than 2% IgA$^+$, and greater than 95% CD20$^+$. IgG-negative B cells were prepared by using biotinylated anti–human IgG (BD PharMingen) and auto-MACS. The resultant IgG-negative B-cell population was less than 3% IgG$^+$. B cells were cultured in RPMI 1640 medium supplemented with 25 mM *N*-2-hydroxyetylpiperazine-*N*′-ethanesulfonic acid (HEPES), 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 5.5×10^{-2} mM β-mercaptoethanol, 100 U/mL penicillin, and 100 U/mL streptomycin (all from Invitrogen, Carlsbad, CA). To stimulate B cells with APRIL and BAFF, the dose of APRIL and BAFF (1, 2, 4, or 8 μg/mL) was first titrated, and it was determined that the doses at 8 μg/mL for APRIL and 4 μg/mL for BAFF were most effective to induce cell proliferation and IgA and IgG production of human peripheral blood B cells in the present system. In some experiments, B cells were treated with heparitinase (10 U/mL) for 10 minutes at 37°C.

Gene transfer of siRNA

TACI siRNA (214802, 5 μg; Ambion, Austin, TX) or control siRNA (4611, 5 μg; Ambion) was transfected into B cells (4×10^6 cells) with the use of the human B-cell nucleofector kit and nucleofector (Amaxa Biosystems, Gaithersburg, MD) and the U-15 program. After 16-hour incubation, cells were subjected to experiments for evaluation of TACI knockdown.

Flow cytometric analysis

After incubation of arbitrary antibody with 2 μg/10^6 cells for 20 minutes on ice, the cells were washed and resuspended in propidium iodide solution and analyzed using FACSCalibur (Becton Dickinson, Mountain View, CA) and associated CellQuest (Becton Dickinson) software. In some experiments, after incubation of 10 ng APRIL-FLAG or BAFF-FLAG with 1×10^6 cells for 30 minutes at 37°C, the cells were washed and incubated with anti-FLAG M2 mAb for 20 minutes on ice. Fluorescein isothiocyanate (FITC)–labeled goat antibody to mouse IgG (Caltag, Burlingame, CA) was used as a secondary antibody. Mouse IgG control antibody was used to evaluate the background.

Proliferation assay

Sixteen hours after transfection with TACI siRNA or control siRNA, B cells were cultured in a 96-well plate (1×10^5/well) with anti-BCR antibodies (anti-Igκ and anti-Igλ, 0.5 μg/mL each), CD40L (2 μg/mL), BAFF (4 μg/mL), APRIL (8 μg/mL), anti-TACI mAb (5 μg/mL), or control mouse IgG2a (5 μg/mL) in the presence or absence of IL-4 (20 U/mL) and TGF-β (1 ng/mL). B-cell proliferation was quantitated by pulsing the cells during the last 18 hours of 72-hour culture with 0.5 μCi (18.5 kBq) per well of [^{3}H] thymidine and using a liquid scintillation beta counter (TopCount NXT; Perkin Elmer, Wellesley, MA).

Detection and quantification of in vitro immunoglobulin secretion

After culture of IgG-negative or IgA-negative B cells in a 96-well plate (1×10^5/well) for 10 days with arbitrary stimulations, the amount of IgG, IgA, or IgM secreted in the culture supernatants was determined by enzyme-linked immunosorbent assay (ELISA) using anti–human Ig (Southern Biotechnology, Birmingham, AL) as the capture antibody, and horseradish peroxidase (HRP)–labeled goat anti–human IgG (ICN Biomedicals) HRP-labeled goat anti–human IgA or HRP-labeled goat anti–human IgM (Sigma-Aldrich) as the detector antibody. After the addition of *p*-nitrophenyl phosphate substrate (Sigma-Aldrich), the amount of IgG, IgA, or IgM was measured by spectrophotometry at 490 nm using a microplate reader (model 550; Bio-Rad, Hercules, CA).

Immunoblot analyses

To prepare whole cell lysates, cells were washed with phosphate-buffered saline (PBS), suspended in 0.5% sodium dodecyl sulfate (SDS) solution, and boiled for 5 minutes. To prepare nuclear extracts, cells were treated with Nuclear Extract Kit (Active Motif). Proteins (5-8 μg) were separated electrophoretically by SDS-PAGE and then were transferred onto an Immobilon-P (Millipore, Bedford, MA) membrane. Immunoblots were probed using arbitrary antibody and developed with HRP-labeled secondary antibody (Amersham Biosciences, Piscataway, NJ). Blotting was visualized and subjected to densitometric analysis with the use of a LumiVision analyzer (Taitec, Saitama, Japan).

Statistical analysis

All data were expressed as mean ± SD. Differences between groups were examined for statistical significance using the paired *t* test. $P < .05$ denoted a statistically significant difference.

Results

TACI and HSPG contribute equally to the binding of APRIL to B cells

To clarify the roles of TACI and HSPG in APRIL-induced B-cell responses, we first examined the dependence on TACI and HSPG in APRIL-binding to human peripheral blood B cells by using an siRNA approach to knock down TACI or treatment with heparitinase to cut the heparan sulfate side chain of HSPG, which is crucial for APRIL binding.[8] An exogenously supplied siRNA for TACI resulted in complete loss of TACI expression at the protein level (Figure 1A) and its cell surface expression (Figure 1B, left panel) compared with control siRNA, whereas the binding of monoclonal antibody (mAb) specific for the heparan sulfate side chain of HSPG was unaffected (Figure 1B, right panel). Similarly, heparitinase-treated B cells showed almost no binding of anti–heparan sulfate mAb (Figure 1B, right panel) but did show unaffected binding of anti-TACI mAb (Figure 1B, left panel). TACI surface expression on B cells was unchanged by treatment with heparitinase for 24, 48, and 72 hours (data not shown). Although BCMA shares the role of APRIL receptor, few or no BCMA-positive cells constituted human peripheral blood B cells, and BAFF-R and CD40 expression patterns did not change by heparitinase or TACI siRNA treatment (Figure S2). Under these conditions, we compared the binding ability of BAFF and APRIL to B cells by using FLAG-tagged recombinant proteins. The proportion of APRIL bound to B cells was equally reduced by the depletion of TACI or the denaturation of HSPG, each of which showed complete loss of its binding (Figure 1C, left panel). On the other hand, TACI knockdown led to reduced binding of BAFF but to no change after denaturation of

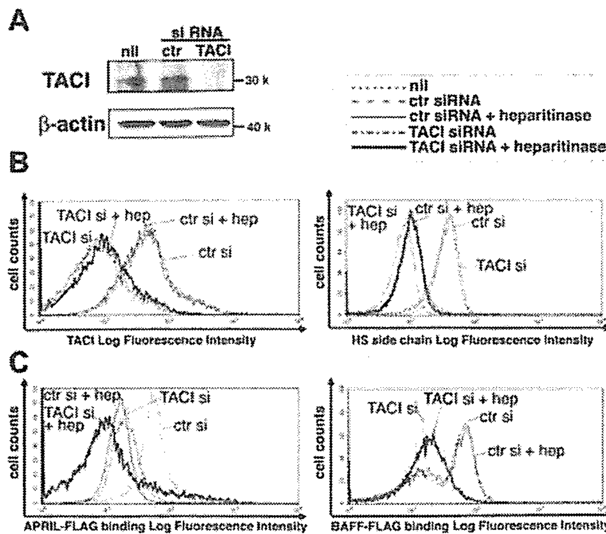

Figure 1. Effects of TACI siRNA and heparitinase on APRIL and BAFF binding to B cells. (A) Down-regulation of TACI by siRNA. Control or TACI siRNA was transfected into human peripheral blood B cells, as described in "Materials and methods," and was subjected to immunoblot analysis probed by polyclonal anti-TACI antibody. β-Actin represents the loading control. (B) Cell surface expression of TACI and HSPG. B cells transfected with TACI siRNA or control siRNA were stained with anti-TACI mAb (11H3; left) or anti–heparan sulfate side chain of HSPG mAb (10E4; right) in the presence or absence of heparitinase (10 U/mL) for 10 minutes at 37°C. (C) Reduced binding ability of APRIL and BAFF by the defect of TACI and HSPG. Cells were treated as in panel B and were stained with FLAG-tagged APRIL (left) or BAFF (right). Stained cells were analyzed by flow cytometry. Ctr indicates control siRNA; hep, heparitinase treatment. Data are representative of 3 independent experiments with similar results.

HSPG (Figure 1C, right panel). These results suggest that HSPG and TACI contribute equally to APRIL binding to B cells.

TACI and HSPG are required for APRIL-induced IgA production, whereas TACI inhibits BAFF-induced B-cell proliferation and production of IgA and IgG

To determine the contribution of TACI and HSPG in APRIL-induced B-cell responses such as cell growth and immunoglobulin CSR, we evaluated cell proliferation, IgA secretion from IgA-negative B cells, and IgG secretion from IgG-negative B cells after treatment with TACI siRNA and heparitinase. B cells with control siRNA showed enhanced B-cell proliferation and secretion of IgA and IgG after stimulation with CD40L, BAFF, and APRIL in the presence of IL-4, TGF-β, and anti-BCR antibodies (Figure 2A, open bars). BAFF-induced B-cell proliferation and secretion of IgA and IgG were significantly enhanced to almost the same level as CD40L by TACI siRNA but not by heparitinase treatment (Figure 2A, lane 5). These results clearly indicate that TACI negatively regulates BAFF-induced B-cell responses. On the other hand, APRIL-induced B-cell responses were almost completely inhibited by heparitinase treatment (Figure 2A, lane 6). Importantly, TACI knockdown did not result in changes in B-cell proliferation, almost completely inhibited IgA secretion, and slightly suppressed IgG secretion by APRIL (Figure 2A, lane 6). To confirm that APRIL-induced IgA and IgG secretion observed here reflects APRIL-induced IgA and IgG CSR, respectively, we counted the number of viable cells and IgM secretion after culture (Table S1), calculated per cell immunoglobulin secretion based on Figure 2A and Table S1, and normalized the levels of secreted IgA and IgG to levels of IgM secretion (Figure 2B). APRIL increased the IgA/IgM ratio,

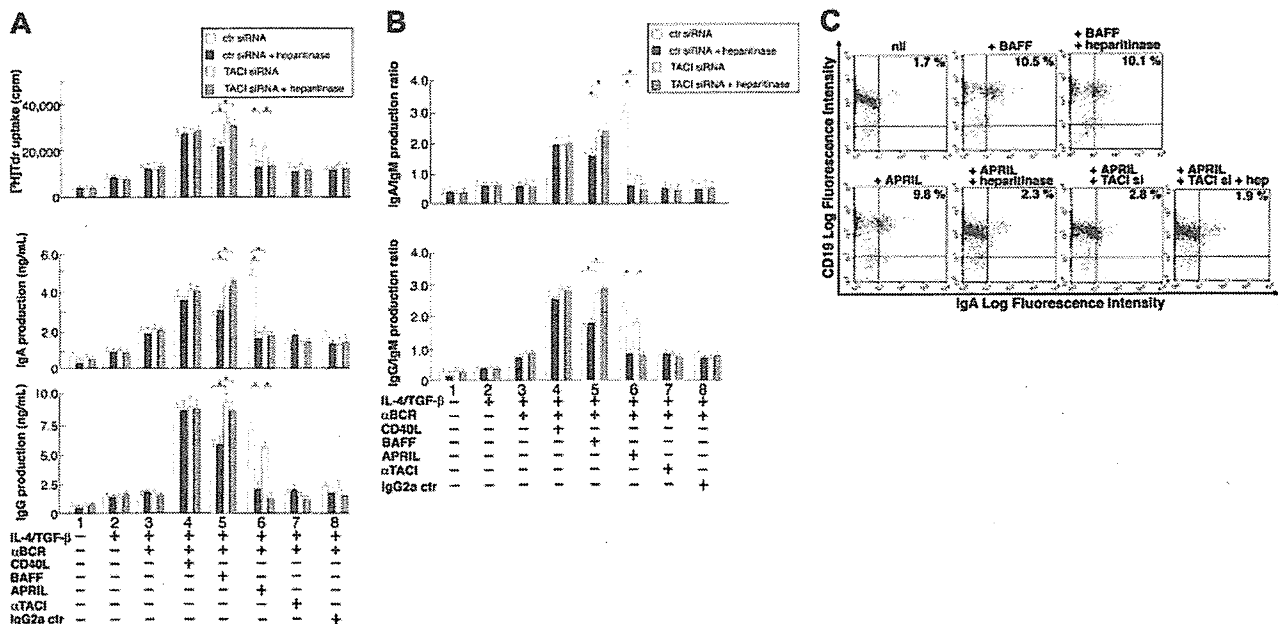

Figure 2. Effects of TACI siRNA and heparitinase on APRIL- and BAFF-induced B-cell responses. (A) Effects of TACI siRNA and heparitinase treatment on B-cell proliferation (top), IgA production (middle), and IgG production (bottom) in response to CD40L, BAFF, APRIL, and agonistic anti-TACI mAb. IgA- or IgG-negative B cells treated with control siRNA, TACI siRNA, or heparitinase (10 U/mL) were cultured with anti-BCR antibodies (anti-Igκ and anti-Igλ, 0.5 μg/mL each), CD40L (2 μg/mL), BAFF (4 μg/mL), APRIL (8 μg/mL), anti-TACI mAb (11H3; 5 μg/mL) or control mouse IgG2a (5 μg/mL) in the presence or absence of IL-4 (20 U/mL) and TGF-β (1 ng/mL). [^{3}H]-Thymidine incorporation in B cells was measured during the last 18 hours of 72-hour culture (top). IgA (middle) and IgG (bottom) secretion were measured by ELISA after 10-day culture. Data are mean ± SD. *$P < .05$. (B) Ratios of IgA/IgM and IgG/IgM production in response to CD40L, BAFF, APRIL, and agonistic anti-TACI mAb. After incubation, as described in panel A, IgM secretion and viable cell number were determined after 10-day culture (Table S1). Per cell IgA, IgG, or IgM production was calculated based on data shown in panel A and Table S1. Then the ratios of IgA/IgM and IgG/IgM were determined. Data are mean ± SD. *$P < .05$. (C) Flow cytometric analysis of IgA-positive B cells. After incubation as described in panel A, cells were stained with PE-labeled anti-CD19 mAb and FITC-conjugated anti-IgA antibody and were analyzed in living cells only. The percentage of CD19$^+$ IgA$^+$ cells is indicated in each plot. Data are representative of 3 independent experiments with similar results.

which was reduced by treatment with TACI siRNA and heparitinase, though APRIL also increased the IgG/IgM ratio, which was reduced by treatment with heparitinase but not by TACI siRNA (Figure 2B, lane 6). In agreement with the data that APRIL-induced IgA CSR and production was abolished by TACI siRNA, flow cytometric analysis showed that 9.8% of untreated B cells were positive for IgA after 48-hour stimulation with APRIL to a level comparable to that of BAFF stimulation (Figure 2C). In contrast, only 2.3% or 2.8% of B cells were positive for IgA after treatment with heparitinase or TACI siRNA, respectively (Figure 2C). It should be noted that agonistic anti-TACI mAb itself failed to elicit B-cell responses (Figure 2A, lane 7). Thus, these results suggest that HSPG is essential for APRIL-induced B-cell responses and that TACI positively regulates APRIL-induced IgA CSR and production but not B-cell proliferation or IgG production. APRIL binding to TACI and HSPG is indispensable for IgA production.

TACI-dependent PKA activation is induced by APRIL but not by BAFF

Nuclear factor-κ B (NF-κB) signaling has 2 pathways, canonical NF-κB1 and noncanonical NF-κB2 pathway, as evidenced by the nuclear translocation of p50/RelA (p65) and p52/RelB complexes,[23,24] respectively. Stimulation with CD40L and BAFF preferentially activates the noncanonical pathway responsible for B-cell responses, such as cell proliferation, immunoglobulin production, and AID-mediated CSR.[25] As shown in Figure 3, NF-κB1 translocation induced by BAFF, APRIL, and agonistic anti-TACI mAb (lanes 3-5) was greatly diminished by TACI siRNA (lanes 15-17) and by heparitinase treatment (lanes 21-23). Although NF-κB2 translocation was induced by BAFF and APRIL (lanes 3-4), the translocation by BAFF but not by APRIL was greatly enhanced by TACI siRNA (lane 15), which was irrelevant to the state of HSPG (lane 21). On the other hand, denaturation of HSPG diminished APRIL-induced NF-κB2 translocation (lane 4 vs lane 10), which was not influenced by TACI siRNA (lane 22 vs lane 16). Moreover, the induction of AID paralleled the behavior of NF-κB2 translocation, indicating that APRIL-induced NF-κB1 translocation is dependent on TACI and that APRIL/HSPG binding is necessary for NF-κB2 translocation and AID induction.

Next, we examined the status of PKA activation because PKA plays a critical role in the posttranslational regulation of AID activity and in the induction of immunoglobulin CSR through the phosphorylation of AID.[26,27] PKA phosphorylation was clearly observed in response to CD40L, BAFF, and APRIL (lanes 2-4). When treated with heparitinase, PKA phosphorylation was blocked in response to APRIL but not to CD40L or BAFF (lanes 8-10). Loss of PKA phosphorylation in TACI knockdown B cells was observed in APRIL stimulation but not in BAFF (lanes 15-16). Thus, impaired APRIL-induced IgA secretion (Figure 2A) in TACI knockdown B cells correlated with impaired PKA phosphorylation but not AID induction or NF-κB2 translocation. These results suggest that APRIL-induced PKA activation requires TACI and HSPG ligation and that TACI may play a pivotal role in APRIL-induced IgA production by regulating AID activity through PKA phosphorylation.

Simultaneous coligation of TACI and HSPG is required for PKA activation and subsequent IgA production

To confirm the mutual cooperation between TACI and HSPG in APRIL-induced IgA production, we tried to mimic the coligation of TACI and HSPG with specific antibodies and their second antibody instead of APRIL ligation. Figure 4 shows that phosphorylation of PKA (Figure 4A) and IgA secretion (Figure 4B) were induced when TACI and HSPG were coligated simultaneously, indicating that APRIL binding to TACI and HSPG is essential for IgA production. We then examined whether the defective PKA activation was sufficient to explain the lack of APRIL-induced IgA secretion in TACI knockdown B cells by testing whether constitutive PKA activity could compensate for the defect of TACI signaling. For constitutive activation of PKA, we used 8-bromo-cAMP, a cell-permeable cAMP analog known to act as a potent inducer of PKA activation.[28] As expected, cAMP administration resulted in PKA phosphorylation even in the absence of TACI (Figure 5A), which in turn recovered APRIL-induced IgA secretion (Figure 5B), indicating the essential role of TACI-mediated PKA activation in APRIL-induced IgA production. Thus, HSPG-mediated AID induction was insufficient for IgA production and TACI-mediated PKA activation was indispensable for APRIL-induced IgA production. These results suggest that the defective secretion of IgA by TACI knockdown B cells was caused by insufficient PKA activation and that TACI and HSPG synergistically mediated a signal for PKA activation and IgA production.

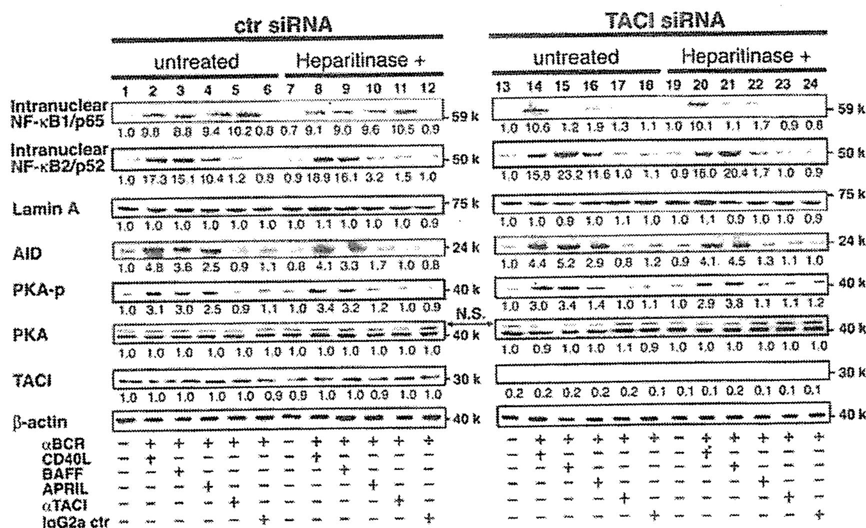

Figure 3. Effects of TACI siRNA and heparitinase on APRIL- and BAFF-induced NF-κB2 and AID expression and PKA phosphorylation. B cells pretreated with control siRNA, TACI siRNA, or heparitinase (10 U/mL) were cultured with anti-BCR antibodies (anti-Igκ and anti-Igλ, 0.5 μg/mL each), CD40L (2 μg/mL), BAFF (4 μg/mL), APRIL (8 μg/mL), anti-TACI mAb (11H3; 5 μg/mL), or control mouse IgG2a (5 μg/mL) in the presence of TGF-β (1 ng/mL). Incubation times differed with individual probes: 30 minutes for NF-κB1/p65 and TACI, 6 hours for NF-κB2/p52, 72 hours for AID, and 45 minutes for PKA phosphorylation. Nuclear extracts and cell lysates were prepared and subjected to immunoblot analysis. Density of each band was analyzed (LumiVision analyzer) and was presented as relative fold of the minimum density in each panel. Data are representative of 3 independent experiments with similar results.

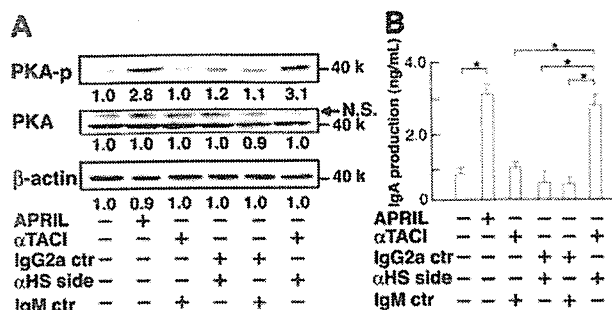

Figure 4. Effects of coligation of TACI and HSPG on PKA phosphorylation and IgA production. (A) IgA-negative B cells were cultured with anti-TACI mAb (11H3), anti–heparan sulfate side chain mAb (10E4), mouse IgG2a, or mouse IgM (5 μg/mL each) for 45 minutes. Then anti–mouse Igκ (1 μg/mL) antibody was added to establish the cross-linking of cell-bound mAbs. APRIL (8 μg/mL) was used as a positive control. After 45-minute incubation, cell lysates were prepared and subjected to immunoblot analysis (B) IgA concentrations in culture supernatant were measured by ELISA after 10 days. Data are mean ± SD. *$P < .05$. Data are representative of 3 independent experiments with similar results.

Discussion

In the present study, we showed that TACI was a positive regulator for APRIL-induced IgA production and a negative regulator for BAFF-induced B-cell responses such as B-cell proliferation and production of IgA and IgG in human peripheral blood B cells in vitro. In addition, we demonstrated that HSPG was essential for APRIL-induced B-cell responses, including the activation of noncanonical NF-κB2 pathway and the induction of AID, whereas TACI contributed less to APRIL-induced B-cell responses except for PKA activation and IgA production.

The positive role of TACI and APRIL in IgA production appears to be important in vivo because TACI and APRIL knockout mice showed reduced levels of serum IgA and deficient IgA responses to mucosally immunized antigens, and old APRIL transgenic mice showed increased levels of serum IgA.[13,14,19,29,30] In addition, defects in TACI have been reported in patients with IgA deficiency and CVID.[16,17] Why is APRIL/TACI rather than BAFF/BAFF-R dominant in IgA production in vivo? The reason might be explained by the localization of IgA-producing cells and TACI-expressing cells. Most IgA antibody is synthesized by mucosal plasma cells associated with lymphoid tissue, gut-associated B cells, and B-1 B cells.[31,32] It has been reported that TACI but not BAFF-R is highly expressed in human small intestine[3,7] and that murine B-1 B cells bind strongly and specifically to APRIL.[30]

We observed that TACI stimulation itself could not induce B-cell activation (Figure 2A); thus, it is unlikely that TACI alone is involved directly in APRIL-induced IgA production. In addition, a previous report described a deficiency of immunoglobulin CSR in TACI knockout B cells after stimulation with APRIL in mice,[29] which is almost similar to the result described here for IgA production with TACI knockdown, in addition to the up-regulation of AID and the mild reduction in IgG. These observations suggest that HSPG plays a major role in APRIL-induced immunoglobulin CSR and that the synergistic ability of TACI is responsible for IgA CSR with HSPG. In this regard, we demonstrated that the coligation of TACI and HSPG by specific mAbs clearly induced IgA production instead of APRIL (Figure 4). However, because HSPG is widely expressed throughout the B-cell lineage as transmembrane proteins such as CD44 and syndecan 1[8,33] and the expression of TACI varies after activation,[34] TACI seems to play a critical role in APRIL-induced IgA production under physiological conditions.

We showed here that although AID was expressed at comparable levels in B cells with TACI and control siRNA (Figure 3), APRIL-induced IgA secretion was impaired by TACI siRNA whereas IgG secretion was slightly reduced compared with siRNA control (Figure 2). It is possible that the dissimilarity in TACI-mediated immunoglobulin responses resulted from differences in the threshold of PKA-mediated phosphorylation that affect AID functionality to induce immunoglobulin CSR. Castigli et al[29] demonstrated the preferential induction of molecular events in IgG CSR rather than IgA CSR in TACI knockout B cells in response to APRIL. Basu et al[26] identified several phosphorylation sites on AID responsible for IgG CSR and demonstrated marked abrogation of IL-4–, TGF-β–, and CD40-induced IgA CSR by PKA inhibition, suggesting that IgA CSR might require more potent PKA activity than IgG CSR. Our finding that activated PKA by a cAMP analog has a capacity sufficient to induce APRIL-induced IgA secretion, despite the absence of TACI (Figure 5), suggests that AID is induced by the binding of APRIL to HSPG and becomes activated after TACI-dependent proper regulation of PKA.

It has been reported that mouse splenic B cells proliferate in response to a form of APRIL that cannot bind HSPG or APRIL in the presence of heparin.[9] However, we could not observe APRIL-induced proliferation of human peripheral blood B cells treated with heparitinase (Figure 2A) and cultured in the presence of heparin (data not shown). Perhaps these results were different because the APRIL splice variant present in mice can bind to mouse BAFF-R though it does not exist in humans and because human APRIL does not interact with human BAFF-R, as reported recently.[35] In addition, it has been reported that TACI knockout B cells do not show IgA CSR and production in response to BAFF in mice.[29] Given that we could not observe an obvious defect in IgA secretion by TACI knockdown B cells in response to BAFF, the different results might be attributed, at least in part, to differences between murine splenic B cells and human peripheral blood B cells.

Although normal humoral responses to TD-Ags and B-cell maturation have been observed in TACI knockout mice, humoral responses to TI-Ags were impaired.[13] B cells responding to TI-Ags reside largely in marginal zone (MZ) B-cell and B-1 B-cell compartments.[36,37] It has been reported that TACI is highly expressed on mouse MZ B cells[34] and that B-1 B cells bind strongly and specifically to APRIL.[30] Thus, it is possible that adequate APRIL binding to TACI and HSPG on these B cells is a more likely explanation of the effects observed in TACI knockout mice. MZ B

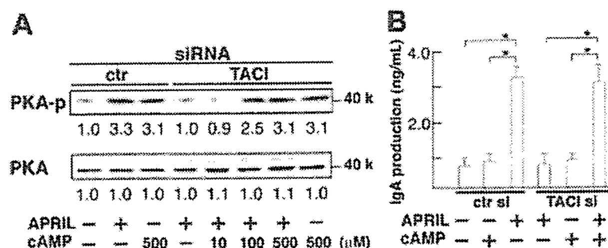

Figure 5. Recovery of APRIL-induced IgA production by activation of PKA in the absence of TACI. Controller TACI siRNA-transfected B cells were cultured with different doses of cAMP analog 8-bromo-cAMP (A; 10, 100 or 500 μM) (B; 500 μM) for 2 hours. Cells were further cultured in the presence of APRIL (8 μg/mL), anti-BCR antibodies (anti-Igκ and anti-Igλ, 0.5 μg/mL each), and TGF-β (1 ng/mL). PKA phosphorylation by immunoblotting (A) or IgA secretion by ELISA (B) was evaluated after 45 minutes or 10 days culture, respectively. Data are mean ± SD. *$P < .05$. Data are representative of 3 independent experiments with similar results.

cells and B-1 B cells can readily undergo CSR from IgM to IgG or IgA in a T cell–independent fashion.[38] Recent studies suggest that T cell–independent CSR requires the interaction of B cells with dendritic cells.[21,39] APRIL secreted from dendritic cells by TI-Ags might enable proper antibody production from MZ and B-1 B cells.

Homozygous and heterozygous mutations in TNFRSF13B are associated with the loss of TACI function, as reported in patients with CVID and IgA deficiency.[16,17] Conversely, strong induction of mature B-cell proliferation, B-cell hyperresponsiveness, lymphadenopathy, and systemic lupus erythematosus nephritis was observed in TACI knockout mice.[13-15] However, only a minority of CVID patients showed signs of autoimmunity or lymphoproliferation.[16] In theory, it could still be possible that HSPG or TACI is expressed on human B cells at levels different from those on murine B cells, reflecting differences in severity. As discussed, TACI is a negative regulator for BAFF-induced B-cell responses and a positive regulator of APRIL-induced IgA production and humoral responses to TI-Ags in collaboration with HSPG. Thus, immunoglobulin deficiency other than IgA observed in CVID patients with TACI defects might have resulted from other CVID disease-specific factors.

In conclusion, the present study identified bilateral regulatory roles of TACI B-cell responses and their importance, especially in IgA production in collaboration with HSPG. These new findings should enhance our understanding of mucosal immune system and humoral responses to TI-Ags.

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Authorship

Contribution: D.S. performed the experiments and wrote the paper; H.H. analyzed the data and participated in the writing of the paper; Y.K. collected the data; H.K. analyzed the data; K.O. contributed vital new reagents; and T.K. designed the research protocol and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Noninvasive and real-time assessment of reconstructed functional human endometrium in NOD/SCID/γ_c^{null} immunodeficient mice

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Human uterine endometrium exhibits unique properties of cyclical regeneration and remodeling throughout reproductive life and also is subject to endometriosis through ectopic implantation of retrogradely shed endometrial fragments during menstruation. Here we show that functional endometrium can be regenerated from singly dispersed human endometrial cells transplanted beneath the kidney capsule of NOD/SCID/γ_c^{null} immunodeficient mice. In addition to the endometrium-like structure, hormone-dependent changes, including proliferation, differentiation, and tissue breakdown and shedding (menstruation), can be reproduced in the reconstructed endometrium, the blood to which is supplied predominantly by human vessels invading into the mouse kidney parenchyma. Furthermore, the hormone-dependent behavior of the endometrium regenerated from lentivirally engineered endometrial cells expressing a variant luciferase can be assessed noninvasively and quantitatively by *in vivo* bioluminescence imaging. These results indicate that singly dispersed endometrial cells have potential applications for tissue reconstitution, angiogenesis, and human–mouse chimeric vessel formation, providing implications for mechanisms underlying the physiological endometrial regeneration during the menstrual cycle and the establishment of endometriotic lesions. This animal system can be applied as the unique model of endometriosis or for other various types of neoplastic diseases with the capacity of noninvasive and real-time evaluation of the effect of therapeutic agents and gene targeting when the relevant cells are transplanted beneath the kidney capsule.

animal model | bioluminescence imaging | endometriosis | menstruation | angiogenesis

Human endometrium lines the uterus and comprises luminal and glandular epithelial cells, stromal fibroblasts, vascular smooth muscle cells, endothelial cells, and immune competent cells. These cell components coordinately participate in the cyclical changes of human endometrium, including proliferation, differentiation, and tissue breakdown and shedding under the influence of estrogen and progesterone during the menstrual cycle. This unique system of cyclic tissue regeneration also depends on the cyclical growth and regression of the blood vessels that supply the endometrium (1). In addition, angiogenesis is deeply involved in the pathogenesis of endometrium-derived disorders such as endometriosis (2). Endometriosis, one of the most common gynecological diseases, is characterized by the presence of functional endometrial-like tissue outside the uterine cavity. It is an estrogen-dependent disorder associated with substantial morbidity; however, the etiology and pathophysiology are not well elucidated (3). To study the physiology of normal endometrium and the pathogenesis of endometriosis, a variety of *in vivo* models using small animals has been developed by using the transplantation of autologous or heterologous endometrial cells/tissues or endometriotic tissues (4).

In the present study, taking advantage of newly developed severe immunodeficient mice, NOD/SCID/γ_c^{null} (NOG) mice (5), we have developed a mouse model that satisfies the following requirements: (*i*) the transplant of human origin is quantitatively and characteristically uniform in each animal, (*ii*) functional and morphological changes characteristic of human eutopic and/or ectopic endometrium are reproduced, and (*iii*) the transplant is assessable for a long term in a noninvasive, real-time, and quantitative manner. By using this model, evidence has been obtained suggesting a previously uncharacterized mechanism underlying the regeneration and remodeling of human endometrium and the pathogenesis of endometriosis.

MEDICAL SCIENCES

Results

Reconstruction of Human Endometrial Tissues in NOG Mice. We first isolated and dissociated endometrial cells mechanically and enzymatically from human cycling endometrium. We transplanted 5×10^5 of these singly dispersed endometrial cells (SDECs) beneath each kidney capsule of ovariectomized (OVX)-NOG mice. Multiple immunological dysfunctions, including cytokine production incapacity and functional incompetence of T, B, and natural killer (NK) cells, may explain the high success rates of xenografts in NOG mice (5). Xenotransplanted NOG mice were subjected to different hormonal treatments (Fig. 1*A*).

Surprisingly, endometrium-like tissues were found in all of the transplanted kidneys of NOG mice ($n = 30$) hormonally treated for 10 weeks (Fig. 1*B*), whereas a very tiny but macroscopically identifiable tissue was reconstituted in only 1 of 12 nonhormonally treated mice (data not shown). Treatment with estradiol (E_2) and progesterone (P_4), or $E_2 + P_4$ treatment, generated a large

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Abbreviations: NOG, NOD/SCID/γ_c^{null}; SDECs, singly dispersed endometrial cells; OVX, ovariectomized; NK, natural killer; E_2, estradiol; P_4, progesterone; $E_2 + P_4$, treatment with E_2 and P_4; Vm, vimentin; αSMA, α-smooth muscle actin; PR, P_4 receptor; PRL, prolactin; BLI, bioluminescence imaging; YFP, yellow fluorescent protein; CBR luc, click beetle red-emitting luciferase; PI, propidium iodide.

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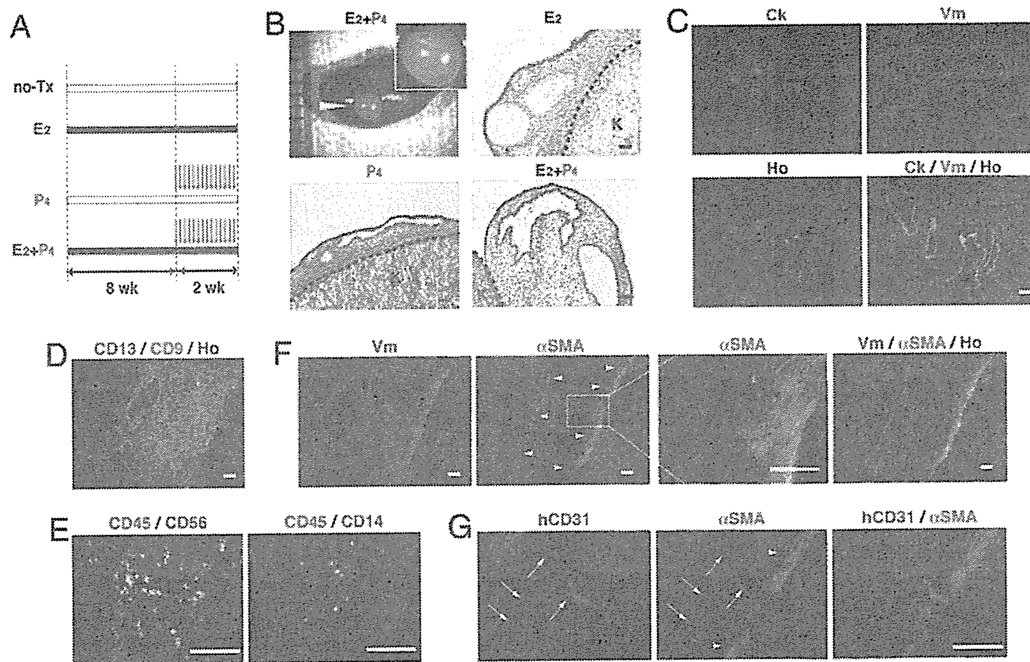

Fig. 1. Reconstruction of human endometrium-like tissue from SDECs. (*A*) Experimental design and hormonal treatment. NOG mice were OVX, xenotransplanted with SDECs, and then treated without (no-Tx) or with s.c. implantation of usually two E_2 pellets alone (E_2) or with a daily s.c. injection of P_4 (red arrows) for the last 2 weeks (P_4) or in combination with E_2 (E_2 + P_4); they were finally nephrectomized 10 weeks after transplantation. (*B*) Macroscopic and microscopic findings of the transplanted site (arrowhead) of NOG mice 10 weeks after xenotransplantation. H&E staining was performed on the transplanted lesion of NOG mice treated with E_2, P_4, or E_2 + P_4 as indicated. The borders between the reconstituted tissue and the mouse kidney (K) are indicated by the dotted lines. (*C*–*G*) Immunofluorescence staining of the endometrial reconstructs in the E_2 + P_4-treated NOG mice by using antibodies against cytokeratin (Ck) and Vm (*C*), CD13 and CD9 (*D*), CD56 or CD14 in combination with CD45 (*E*), Vm and α-SMA (*F*), or human CD31 (hCD31) and α-SMA (*G*), followed by Hoechst (Ho) staining. (*F*) Arrows point to the regions prominently immunoreactive for α-SMA. A small box marks a region shown at higher magnification in the adjacent panel as indicated. (*G*) Arrows and arrowheads indicate $hCD31^+/\alpha$-SMA^+ cells and $hCD31^-/\alpha$-SMA^+ cells, respectively. (Scale bars: 100 μm.)

cystic mass (Fig. 1*B*, arrowhead) with a fine surface vasculature (Fig. 1*B Inset*). Hematoxylin and eosin (H&E) staining demonstrated that well delineated glands and stroma were present in the transplanted lesions dissected from E_2- or E_2 + P_4-treated mice (Fig. 1*B*). Furthermore, the growth of the endometrial transplants together with the enlargement of the uterus were E_2 dose-dependent [supporting information (SI) Fig. 6].

Immunofluorescence studies revealed that the reconstructed tissues, but not the mouse kidney, were stained exclusively with anti-human vimentin (Vm) antibody (clone V9) (Fig. 1*C*). As V9 can recognize only human Vm, the reconstructed tissue was clearly of human origin. In addition, the stroma was positive for CD10 (data not shown) and CD13, both endometrial stromal cell markers (6), whereas the glandular structure was positive for cytokeratin and CD9, both epithelial markers (6) (Fig. 1 *C* and *D*). Human endometrial tissue and decidualized endometrium in pregnancy contain large numbers of CD45-positive leukocytes, the vast majority of which are $CD56^+$ NK cells with the other populations being $CD14^+$ macrophages and T cells (7). In agreement with this profile, $CD45^+$ cells were abundant in the reconstructed tissue, and the proportions of $CD56^+$ and $CD14^+$ cells, both of human origin, were similar to those in the eutopic endometrium (Fig. 1*E*).

Expression of α-smooth muscle actin (αSMA) is ubiquitously prominent in the uterine myometrium but is mainly confined to vascular smooth muscle cells in the endometrium (8). In the Vm-positive regenerated tissue, αSMA antibody preferentially and potently reacted with two distinct regions: one adjacent to the mouse kidney parenchyma and the other beneath the serous membrane (Fig. 1*F*, arrowheads). These two regions mainly consist of αSMA-positive spindle-shaped fibroblastic cells morphologically similar to myometrial cells (Fig. 1*G*). The vast majority of these densely populated αSMA^+ cells did not colocalize with $CD31^+$ endothelial cells (Fig. 1*G*, arrowheads). In the same area, however, some αSMA^+ cells were present along with cells positive for CD31, an endothelial cell marker (Fig. 1*G*, arrows). These results collectively suggest that some, but not most, αSMA^+ cells contribute to the formation of the vessels, presumably as pericytes. Thus, the reconstituted tissue possessed hierarchical architecture distinctly composed of endometrium- and myometrium-like layers (Fig. 1*G*), closely resembling the normal uterine structure at the interface between endometrium and myometrium. Absence of αSMA^+ cells in SDECs (data not shown) indicated that they did not contain differentiated myometrial cells, which may account for the lack of spiral arterioles in the reconstituted endometrial tissues, because spiral arteries arise from the myometrium. Furthermore, given the potential of endometrial stroma for differentiation into smooth muscle (9), it is likely that the generation of myometrial-like components may be caused by the transdifferentiation of SDECs into smooth muscle cells. Alternatively, it also remains possible that SEDCs include a population of undifferentiated smooth muscle precursor cells. Taken together, SDECs have the potential to regenerate the endometrium ectopically with tissue polarity, glandular structures, and endometrial cell components.

Neovascularization of the Reconstructed Endometrium by Human–Mouse Chimeric Vessels. We further investigated the vascular components of the E_2 + P_4-exposed endometrial reconstruct. As shown in Fig. 2 *A*, human $CD31^+$ (arrows) or mouse $CD31^+$ (arrowheads) endothelial cells coexisted abundantly in the reconstructed tissue in E_2 + P_4-treated NOG mice; however, they did not appear to anastomose with each other (Fig. 2*A Right*).