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# BAFF supports human B cell differentiation in the lymphoid follicles through distinct receptors

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**Abstract**

**B cell-activating factor of the tumor necrosis factor family (BAFF/BLys) plays a critical role in B cell survival and immune responses through its three receptors: BAFF receptor (BAFF-R/BR3), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) and B cell maturation antigen (BCMA). Using specific antibodies, we have investigated the expression of BAFF-R on human tonsillar B cells and their functional roles in naive and germinal center (GC) B cell differentiation. Our studies show that BAFF-R is the dominant receptor on naive B cells. However, three receptors are differentially modulated during *in vitro* GC-B cell differentiation. BAFF-R expression increased initially and then decreased with a corresponding induction of TACI and BCMA expression during differentiation to plasma cells (PCs). Consistently, blocking of BAFF-R alone with specific mAb inhibited GC-B cell proliferation and PC generation in the early period of their differentiation, whereas depletion of BAFF with TACI-Ig exhibited consistent inhibition throughout the differentiation. Finally, histological and molecular analyses of human tonsil tissue revealed that follicular dendritic cells produce BAFF. In conclusion, BAFF in the GC plays an important role through more than one receptor, and the three known receptors are differentially modulated as GC-B cells differentiate to PCs.**

## Introduction

In the humoral immune response, a highly specific protective antibody is produced by the process of germinal center (GC) reaction (1, 2). Following the persistent B cell receptor stimulation by antigen, GC-B cells rapidly expand by proliferation and differentiate into Ig-secreting plasma cells (PCs) or memory B cells (2, 3). Follicular dendritic cells (FDCs) play an essential role in GC reaction. GC-B cells cannot survive or expand in the absence of FDC (4, 5). FDCs have a crucial co-stimulatory function by preventing apoptosis of GC-B cells. However, the molecules that mediate the anti-apoptotic effect have not been identified.

Recently, studies on the anti-apoptotic B cell-activating factor of the tumor necrosis factor family (BAFF) and its receptors have revealed their critical function in B cell maturation (6–8), immune response (9) and the pathogenesis of autoimmune disease (10–13). In BAFF-deficient mice, the mature B cell population was severely reduced although T and B cell development in the thymus and bone marrow appeared to be normal (14–16). Furthermore, GC reaction and humoral immune responses are impaired following antigen stimulation (17, 18). These results imply that B cells in the peripheral lymphoid follicle are the main target cells for BAFF. Conversely, BAFF transgenic mice have an increased number of peripheral B cells and develop autoimmune diseases such as systemic lupus erythematosus (SLE) and Sjogren's syndrome (7). BAFF appears to play a critical role in maintaining B cell homeostasis in the physiological state.

BAFF has been shown to bind to three receptors: BAFF-R (19, 20), transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) (21, 22) and B cell maturation antigen (BCMA) (15). Two of these receptors, BCMA and TACI, also bind a closely related cytokine, a proliferation-inducing ligand (APRIL) (23). Although all three receptors bind to BAFF, BAFF-R appears to be the most crucial receptor for mature B cell survival and homeostasis in the peripheral lymphoid organs *in vivo*. A/WySnJ mice,

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containing a mutation in BAFF-R, exhibit a phenotype that is similar to BAFF-deficient mice (16, 24). BCMA-deficient mice exhibit normal numbers of mature B cells (16, 25) and impaired the survival of long-lived PCs (26). TACI-deficient mice exhibit increased numbers of mature B cells, developing lymphadenopathy, splenomegaly and autoimmune disease (27, 28). Although *in vivo* experimental models have clearly demonstrated the essential role of BAFF, the functions of the individual receptors are not clear. Hence, we have investigated the function of the BAFF-R in our *in vitro* experimental model that mimics the *in vivo* GC reaction (3, 29).

We show that naive B cells express BAFF-R in higher density than GC-B cells. BAFF-R is a dominant receptor for the survival of the activated naive B cells. However, BAFF plays an essential role through three differentially modulated BAFF-Rs in supporting differentiation of GC-B cells to PCs. In addition, FDCs in the GC produce BAFF.

## Methods

### *Antibodies*

Preparation and specificity of the mouse anti-human BAFF (clone A11.C3.4), anti-human BAFF-R (clone 9.1), anti-human TACI (clone A1G11.4.5 and B10C10.5) and hamster anti-human BCMA (clone C4E2) mAbs were described previously (30, 31). These mAbs were biotinylated following manufacturer's instruction (NHS-LC-Biotin, Pierce Biotechnology, Inc. Rockford, IL, USA). PE-conjugated anti-IgD, FITC-conjugated anti-CD77, allophycocyanin-conjugated anti-CD38, PE-conjugated anti-CD3, FITC-conjugated isotype control (clone AI12-2) and PerCP-Cy5.5-conjugated streptavidin or anti-CD20 were purchased from Pharmingen (San Diego, CA, USA). FITC-conjugated anti-PCs (clone VS38c), dendritic reticulum cell-1 (DRC-1: clone R4/23) and PE-conjugated isotype control (DAK-GO1) were purchased from DAKO (Carpinteria, CA, USA). Other antibodies used in this paper were described previously (32).

### *Reagents*

Cytokines used were IL-2 (Hoffman-La Roche, Inc., Nutley, NJ, USA), IL-4 (a generous gift from Schering-Plough Schering Corporation, Union, NJ, USA) and IL-10 (R&D Systems, Minneapolis, MN, USA). Myc-BAFF was produced as described previously (19). Soluble human CD40 ligand (CD40L) was generously provided by R. Armitage (Amgen Inc., Seattle, WA, USA).

### *Immunohistological and flow cytometry staining*

Frozen sections of human tonsils or the cytospin preparation of human FDC clusters were fixed in cold acetone and stained with corresponding specific mAbs to BAFF-Rs as described in Fig. 1 followed by proper FITC- or PE-conjugated secondary antibodies. Tonsil sections were further stained with anti-IgD–PE or anti-PC–FITC antibody to define B cell sub-populations. For detecting BAFF in FDCs, the cytospin preparations of FDC clusters were incubated with 4′,6-diamidino-2-phenylindole (DAPI) for nuclear counter-staining. The slides were stained with anti-BAFF mAb, DRC-1 mAb or control mAb followed by FITC-conjugated goat anti-mouse Ig. Flow cytometry staining of tonsillar mononuclear cells, cultured GC-B cells or primary FDCs was performed as described in corresponding figure legends following standard protocol (3). Samples were analyzed with FACSCalibur and CellQuest-Pro program (Becton Dickinson, San Jose, CA, USA). At least 5000 cells from each sub-population were collected for data analysis. Specific mean fluorescence intensity (MFI) was calculated by subtraction of fluorescence value of the controls stained with isotype-matched Ig.

### *Culture of B cell subsets*

GC-B cells were purified from tonsillar B cells and cultured as described previously (3) (29). The purity of GC-B cells was >95%, as assessed by the expression of $CD20^+CD38^+$. Briefly, GC-B cells ($2 \times 10^5$ cells per well) were co-cultured with irradiated HK cells ($2 \times 10^4$ cells per well, 5000 rad) in 24-well plates in the presence of a combination of cytokines and blocking reagents as described in figure legends. The concentrations of cytokines used were as follows: IL-2 (30 U $ml^{-1}$), IL-4 (50 U $ml^{-1}$), IL-10 (50 ng $ml^{-1}$) and CD40L (100 ng $ml^{-1}$). The cell recovery was determined by trypan blue exclusion assay. $CD20^+CD38^{\pm}IgD^+$ naive B cells were isolated from high-density B cells by negative selection using anti-CD38 antibody and by positive selection using anti-IgD antibody (32). The purity of naive B cells was >94% by the expression of $CD20^+CD38^{\pm}IgD^+$. The culture medium was Iscove's modified Dulbecco's medium (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% FCS (Life Technologies, Inc., Grand Island, NY, USA), 2 mM glutamine, 100 U $ml^{-1}$ penicillin G and 100 μg $ml^{-1}$ streptomycin (Irvine Scientific). Naive B cells were cultured in triplicates in 96-well plates in the presence of anti-μ (10 μg $ml^{-1}$) for 72 h and pulsed with 0.5 μCi of [$^3$H]thymidine ([$^3$H]TdR: 20 Ci $mM^{-1}$; Dupont NEN, Boston, MA, USA) during the last 16 h culture period. [$^3$H]TdR incorporation was measured by a liquid scintillation counter (Rackbeta, LKB Instruments, Houston, TX, USA). Results were expressed as the mean counts per minute ± SD of triplicate cultures.

### *Isolation of primary FDCs and reverse transcription–PCR*

Human tonsillar FDCs from various donors were isolated and cultured in 10% FCS RPMI media as described previously (4). An FDC line from one donor was selected after screening for *in vitro* GC-B cell supporting activity and named HK. HK cells of passage 2–7 were used for the experiments. Total RNA was extracted from FDCs, HL-60 cells and tonsillar T cells using the RNeasy Protect Mini kit (Qiagen, Valencia, CA, USA). A 1-μg aliquot of RNA was transcribed using oligo dT and Moloney murine leukemia virus reverse transcriptase (M-MLV RT) (Invitrogen-GIBCO, Carlsbad, CA, USA). Complementary DNA was amplified in a 25 μl reaction mixture containing 200 μM of each dNTP, 500 nM of primers and 2.5 U *Taq* polymerase. Amplification of each cDNA sample was performed by the optimized cycles: denaturation at 94°C for 1 min, annealing at 57°C for 1 min and extension at 72°C for 1 min. Amplified PCR products were separated on 1.5% agarose gel and visualized by ethidium bromide staining. Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to normalize each PCR product for

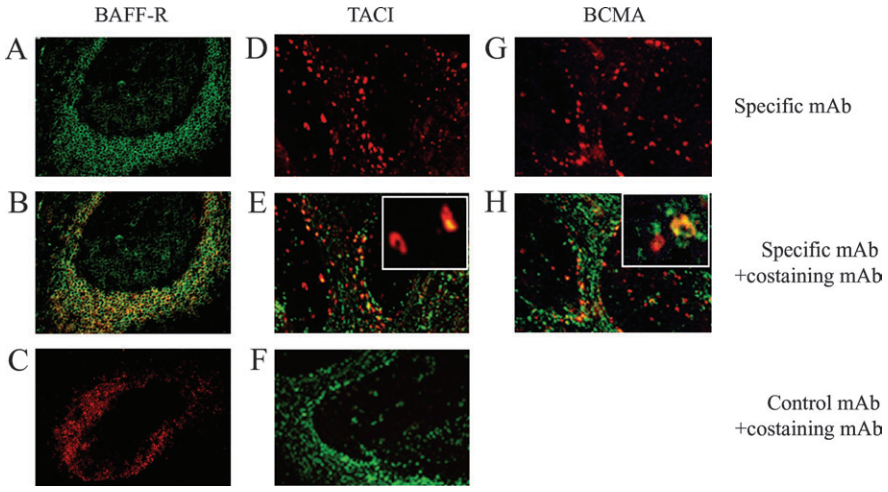


**Fig. 1.** Expression of BAFF-R, TACI and BCMA in human tonsillar tissue. The frozen sections from human tonsil were stained with anti-BAFF-R mAb (A and B, green), anti-TACI mAb (D, E and inserts, red), anti-BCMA mAb (G, H and insert, red) and the corresponding control antibodies (C, green; F, red). Anti-IgD–PE (B and C, red) and anti-PC–FITC (E, F, H and inserts, green) were co-stained to identify B cell sub-populations. Original magnification ×100 (inserts ×400).

equal sample loading. The primer pairs used are as follows—BAFF: forward, 5′-GGAAGGAAAGCCCCTCTGTCC-GATC-3′, reverse, 5′-CTGGACCCTGAACGGCACGCTTATT-3′; GAPDH: forward, 5′-CCCTCCAAAATCAAGTGGGG-3′, reverse, 5′-CGCCACAGTTTCCCGGAGGG-3′.

### *Statistical analysis*

Statistical significance of differences was determined by Student's *t* test or Wilcoxon signed rank test using Prism software (GraphPad Software, Inc., San Diego, CA, USA); $P < 0.05$ was considered significant.

## Results

### *Expression of the BAFF-Rs in tonsillar tissue and on B cell sub-populations*

To explore the *in vivo* expression of BAFF-Rs, tonsillar tissue sections were stained with mAbs specific to BAFF-R, TACI and BCMA. Anti-IgD mAb was co-stained to distinguish the B cell sub-populations. BAFF-R was expressed in B cells in the mantle zone and GC, but not in T cell zone outside the GC (Fig. 1A and B). Compared with BAFF-R, TACI and BCMA were expressed in a few scattered cells throughout the tonsil section without specific sub-zonal staining pattern (Fig. 1D–G and H). The cells expressing TACI revealed a typical morphology of PCs with enlarged cytoplasm and eccentric nucleus. Expression of TACI on PCs was further confirmed by co-staining with a PC marker (anti-PC, yellow color: Fig. 1E, insert) (33). BCMA exhibited a staining pattern similar to that of TACI (yellow color: Fig. 1H, insert). However, not all anti-PC-positive cells express TACI or BCMA (red color: Fig. 1E and H, inserts). The stainings are specific because no expression was observed in tonsillar sections stained with corresponding control antibodies (Fig. 1C and F).

To assess BAFF-R expression quantitatively, mononuclear cells were isolated from tonsillar tissues and examined by four-color flow cytometry analysis (Fig. 2). Among the $CD3^-$ cell fraction, three B cell sub-populations were defined by surface expression of CD38 and IgD: $IgD^+CD38^{\pm}$ naive B cells, $IgD^-CD38^{\pm}$ memory B cells and $IgD^-CD38^+$ GC-B cells (3, 34). GC-B cells were further divided into centrocytes and centroblasts by CD77 expression (35, 36). The flow cytometry analysis results from four donors are summarized in Table 1. Naive B cell subset ($IgD^+CD38^{\pm}$) showed the highest BAFF-R expression (MFI: 173.1) among tonsillar B cell subsets. Although lower than naive B cells, BAFF-R expression of memory B cells was higher than GC-B cells (MFI: 137.7 versus 74.2). Within the GC-B cells, BAFF-R expression of $CD77^-$ centrocytes was higher than $CD77^+$ centroblasts (MFI: 104.8 versus 65.9). For TACI expression, $IgD^-CD38^{\pm}$ memory B cell subset (PC precursor) showed a low but significant level of surface expression (MFI: 12.4). Interestingly, there were two sub-populations in memory B cells in terms of TACI expression. One-third of memory B cells revealed higher TACI expression, whereas the remaining two-third showed negligible TACI staining. This result is consistent with recent report that sub-population of non-GC type tonsillar B cells express TACI (31). The surface expression of BCMA was not significantly higher than the background staining with control mAb in all B cell subsets (Fig. 2, Table 1). The expression of BAFF-Rs on $IgD^-CD38^{++}$ PCs by FACS analysis revealed broad distributions without distinct peaks (data not shown). This observation is consistent with the immunofluorescent staining study, which showed that only a portion of the anti-PC-positive cells was TACI/BCMA positive. Since PCs are not a single homogeneous population but consist of heterogeneous sub-populations of various maturation stages (33), this result suggested a differential expression of BAFF-Rs among PC sub-populations.

### *BAFF-R is a dominant receptor to mediate BAFF activity in naive B cells*

The addition of BAFF increased cellular proliferation when naive B cells were stimulated by anti-μ or *Staphylococcus aureus*

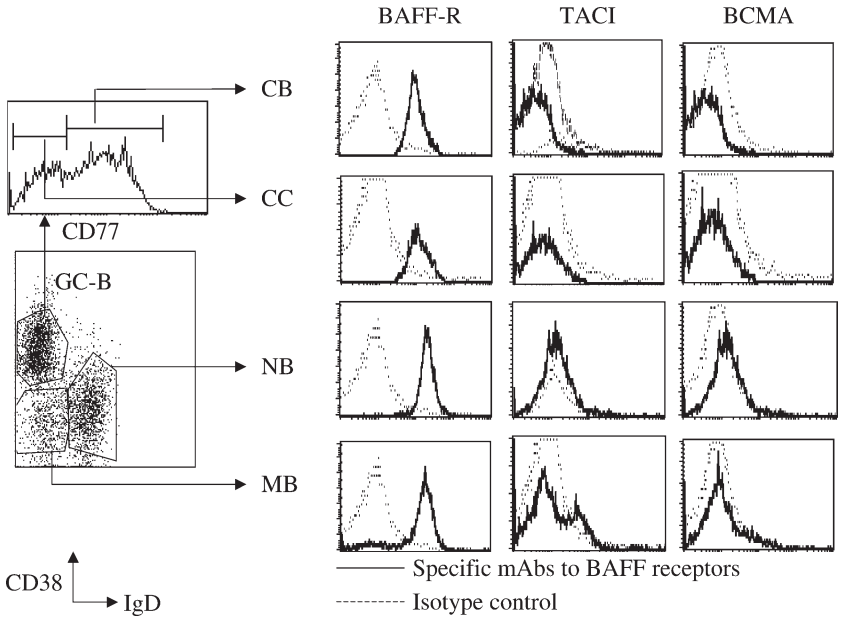


**Fig. 2.** Expression of BAFF-R, TACI and BCMA in human tonsillar B cell subsets. Tonsillar mononuclear cells were freshly isolated as described in Methods and stained with biotin-conjugated mAbs to BAFF-R, TACI or BCMA then, followed by PE-cy5.5-conjugated streptavidin, anti-CD3–PE, anti-CD38–allophycocyanin and anti-IgD–FITC or anti-CD77–FITC. Samples were analyzed with FACSCalibur and CellQuest-Pro program. B cell population was defined by the combination of forward scatter, side scatter and $CD3^-$ gates. For each plot, the solid and broken lines represent the fluorescence of cells stained with the specific and control antibody, respectively. One representative result is shown and the results from four donors are summarized in Table 1 (CB, centroblasts; CC, centrocytes; GC-B, germinal center B cells; NB, naive B cells; MB, memory B cells).

**Table 1.** Expression of BAFF-R, TACI and BCMA in B cell subsets[a]

| Tonsillar B cell subsets | BAFF-R ($n = 4$) | TACI ($n = 4$) | BCMA ($n = 3$) |
|---|---|---|---|
| Naive B ($IgD^+CD38^\pm$) | 173.1 (31.6) | 5.8 (3.4) | 6.0 (2.3) |
| GC-B ($IgD^-CD38^+$) | 74.2 (21.1) | 0.7 (1.0) | 1.1 (2.0) |
| Centrocytes ($IgD^-CD38^+CD77^-$) | 104.8 (15.8) | 1.7 (1.3) | 1.1 (1.5) |
| Centroblasts ($IgD^-CD38^+CD77^+$) | 65.9 (20.2) | 0.0 (0.0) | 0.0 (0.0) |
| Memory B ($IgD^-CD38^\pm$) | 137.7 (17.1) | 12.4 (10.4) | 4.6 (2.1) |

[a]Human tonsillar B cell subsets was defined by flow cytometric analysis as described in Fig. 2. Expression of BAFF-Rs was quantified by staining with biotin-conjugated anti-BAFF-R, anti-TACI or anti-BCMA mAb followed by streptavidin–PerCP-Cy5.5. MFI was determined by subtraction with the isotype control value and then averaged for corresponding subsets (numbers in parentheses represent standard deviation).

Cowan 1 Bacteria (37, 38). To investigate whether the co-stimulatory effect is mediated through BAFF-R, neutralizing anti-BAFF-R mAb or TACI-Ig was added to the anti-μ-stimulated naive B cells (Fig. 3). The addition of the anti-BAFF-R mAb completely neutralized the co-stimulatory effect of BAFF. The blocking effect of BAFF-R was at the same level as that of depletion of BAFF by TACI-Ig, suggesting that BAFF signaling transduced mainly through BAFF-R in naive B cells. The blocking activity of anti-BAFF-R mAb is specific because isotype-matched Ig did not inhibit co-stimulation. Anti-BAFF-R mAb did not bind TACI or BCMA (30). This result is consistent with the intense expression of BAFF-R and very low expression of TACI and BCMA on naive B cells as shown in flow cytometry analysis (Table 1). Therefore, BAFF-R is a dominant receptor that mediates BAFF activity in naive B cells.

### *Expression of the receptors for BAFF is modulated during GC-B cell differentiation in vitro*

GC-B cells differentiate into PCs in the *in vitro* culture that mimics *in vivo* GC reaction (3, 29). As shown in Fig. 4(A), GC-B cells were cultured in the presence of HK cells, CD40L, IL-2 and IL-10 for 11 days (29). At day 2, $CD20^+CD38^+CD44^-CD77^+$ centroblasts became $CD20^+CD38^\pm CD44^+CD77^-$ centrocytes and started to differentiate into $CD20^-CD38^{++}$ IgG-secreting PCs at day 4. By the 11th day of the culture, the viable cell number increased to 800% of the initially seeded cell number (from $2 \times 10^5$ to $16 \times 10^5$) accompanied by a robust secretion of IgG. Such an increase in the cell number and IgG secretion represents actual generation of PCs.

To explore the role of BAFF and its receptors in GC-B cell differentiation to PCs, we examined the expression of BAFF-Rs in the course of GC-B cell differentiation. BAFF-R expression was transiently up-regulated in the total population at day 2 then down-regulated at day 4 through day 11 (Fig. 4B). In contrast, TACI expression started to increase on day 2 and sustained a high level through day 11. BCMA expression appeared to be induced, similar to TACI, but the MFI was less evident.

We next investigated whether these changes in the expression profiles of each BAFF-R were related to the activation status of GC-B cells or differentiation stage of GC-B cells to PCs. To address this, the receptor expressions in

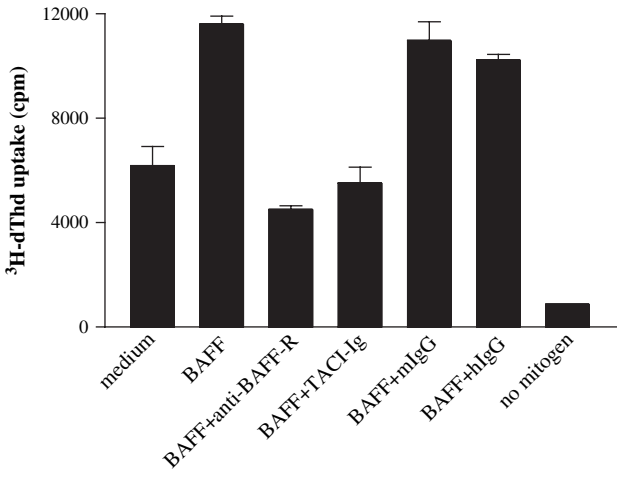


**Fig. 3.** Anti-BAFF-R mAb inhibits BAFF-mediated proliferation of naive B cells *in vitro*. Naive B cells ($1 \times 10^5$ cells ml$^{-1}$) were cultured with anti-μ, IL-2 and IL-10 in the presence or absence of myc-BAFF (100 ng ml$^{-1}$), anti-BAFF-R, TACI-Ig or corresponding control antibodies (all 10 μg ml$^{-1}$) as indicated. After 2 days of culture, cellular proliferation was measured by pulse labeling the cells with 0.5 μCi [$^3$H]TdR 16 h before harvesting. The results represent mean counts per minute ± SD of triplicate cultures (no mitogen represented background dThd uptake in the culture without anti-μ). One representative data from three independent experiments is shown.

generating PCs and PC precursors were examined (Fig. 4C). The expression of BAFF-R on CD20$^-$CD38$^{++}$ PC subset was about three times lower than that of CD20$^+$CD38$^\pm$ subset (PC precursor). The minor increase of BAFF-R expression in B cell sub-population gated as PCs at day 2 may be derived from the contaminating CD20$^+$CD38$^\pm$ B cells with increased BAFF-R expression. The number of CD20$^-$CD38$^{++}$ PCs was <3% of the total cell population and there was no detectable IgG in the culture supernatant at day 2. Compared with BAFF-R, TACI expression revealed a different pattern. TACI expression was up-regulated on CD20$^+$CD38$^\pm$ PC precursors at day 2, reaching a peak at day 4, while TACI expression on CD20$^-$CD38$^{++}$ PCs increased at day 4 and sustained through day 11. TACI staining was consistently higher in CD20$^-$CD38$^{++}$ PCs than in CD20$^+$CD38$^\pm$ PC precursors in four independent experiments. There was a definite increase in the cell number of the BAFF-R$^{low}$-TACI$^{high}$ sub-population as PCs were generated. Viable cell numbers of each subset were calculated by multiplying the percentage of cells with high or low expression of each receptor by the total viable cell number (Fig. 4D). The increase in the cell number was almost equivalent to that of the CD20$^-$CD38$^{++}$ PC number generated (Fig. 4A). A similar increase in TACI-expressing cells did not occur in the control culture with IL-4, where PCs were not generated (data not shown) (29). Taken together, it appears that BAFF-R expression increased in the CD20$^+$CD38$^\pm$ B cell subset in the early stage of GC-B cell differentiation. However, TACI expression appears to increase in the later stage of differentiation when CD20$^+$CD38$^\pm$ PC precursors differentiate into CD20$^-$CD38$^{++}$ IgG-secreting PCs. Expression of BCMA on the surface of cultured GC-B cells appears to be small but significant showing similar modulation to TACI (Fig. 4).

### *BAFF plays an important role during GC-B cell differentiation through different receptors*

Since the differential expression of BAFF-Rs was correlated with differentiation of GC-B cells to PCs, we investigated whether the differential modulation of BAFF-Rs is functionally relevant to GC-B cell differentiation to PCs. We first developed a method to isolate sub-populations expressing different BAFF-Rs. Freshly isolated GC-B cells were first cultured either with IL-10 or with IL-4 in the presence of IL-2, CD40L and HK cells at day −4 (Fig. 5A). After 4 days of culture with IL-10, 73% of cells became CD20$^+$CD38$^\pm$ PC precursors and 21% became CD20$^-$CD38$^{++}$ PCs (Fig. 5A, day 0). Next, these two subsets were separated and cultured with IL-2 and IL-10 for an additional 4 days. CD38$^{++}$ PCs (BAFF-R$^{low}$-TACI$^{high}$) continued to secrete Ig without phenotypic changes in the secondary culture (Figs 5A upper panel at day 4 and B), while CD38$^\pm$ (BAFF-R$^{high}$-TACI$^{low}$) PC precursors differentiated into Ig-secreting CD38$^{++}$ PCs (BAFF-R$^{low}$-TACI$^{high}$; Fig. 5A, middle panel at day 4 and 5B). In the parallel culture with IL-4 in place of IL-10, CD38$^\pm$ cells (BAFF-R$^{high}$-TACI$^{low}$) were cultured with IL-4 continuously to ensure that PCs were not generated (Fig. 5A, lowest panels and 5B).

As shown in Fig. 5(C), the addition of TACI-Ig to deplete BAFF in the initiation of secondary culture inhibited PC generation from CD38$^\pm$ PC precursors by 42.7%, compared with that in the culture with control-Ig, demonstrating the crucial role of BAFF in the differentiation of GC-B cells to PCs. The inhibition by anti-BAFF-R mAb, however, was less than TACI-Ig (20.1% versus 42.7%; $P = 0.009$). The addition of anti-BAFF-R mAb did not inhibit the expansion of CD38$^{++}$ PCs, while TACI-Ig did (−5.1% versus 17.3%; $P = 0.027$). These results suggest that the receptors other than BAFF-R (i.e. TACI or BCMA) are required for BAFF signaling in PC generation from GC-B cells. The relatively small inhibition of PC generation can be attributed to low proliferation activity of mature PCs. On the other hand, the addition of anti-BAFF-R mAb or TACI-Ig in the culture with IL-2 and IL-4 where CD38$^\pm$ PC precursor cells proliferated but did not differentiate to PCs showed comparable levels of inhibition (27% versus 23%; $P = 0.48$). This data suggests that BAFF-R is a major receptor for BAFF signaling in CD38$^\pm$ PC precursor generation but not in PC expansion. These results are consistent with previous flow cytometry results of BAFF-R modulation on differentiating GC-B cells (Fig. 4).

### *FDC/HK cells produce BAFF*

Since GC-B cells expressed receptors for BAFF and the recovery of cultured GC-B cells was inhibited by BAFF depletion, we investigated the cellular source of BAFF within the GC.

We isolated fresh FDCs from four donors and measured BAFF expression. In addition, an FDC line, HK, was mainly used for *in vitro* experiments (Fig. 6A and B). To remove B cells from FDC clusters, FDCs were cultured *in vitro* for 2–5 passages. In reverse transcription (RT)–PCR experiments, a specific band for BAFF was amplified from all four primary FDC samples as well as from HL-60 cells; however, no band was amplified from negative control T cells (Fig. 6A). The specificity of the result was verified by sequencing the

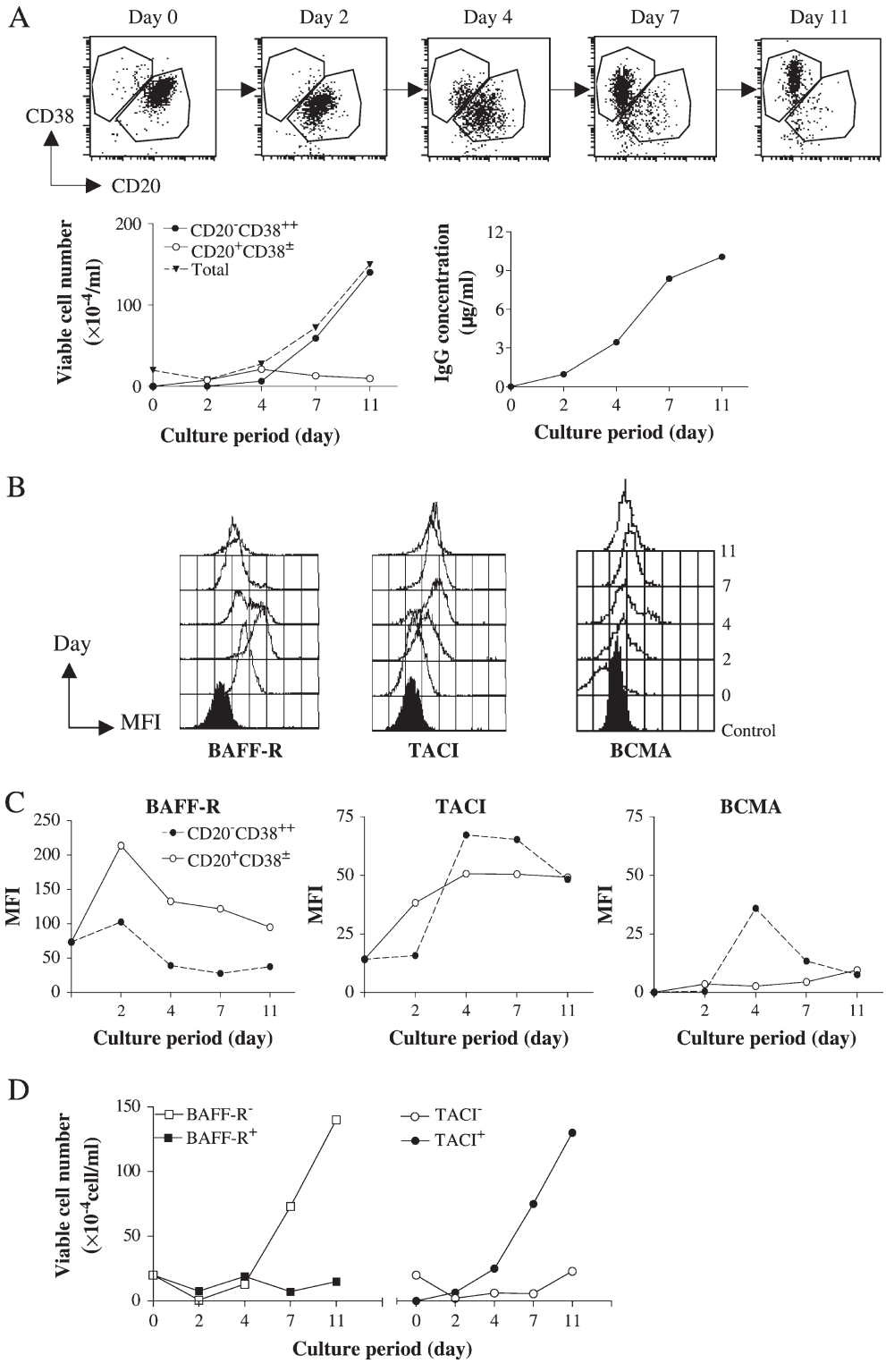


**Fig. 4.** The differential expression of BAFF-Rs during GC-B cell proliferation and differentiation *in vitro*. GC-B cells were cultured in the presence of HK cells, CD40L, IL-2 and IL-10 for 11 days. At the end of indicated culture periods, cells were counted and stained with anti-CD38, anti-CD20 mAbs and biotin-conjugated anti-BAFF-R, anti-TACI or anti-BCMA mAbs to quantify BAFF-R expressions in each subset. (A) Flow cytometry profiles (upper row), the viable cell number (lower left) and IgG concentration in the supernatant (lower right). Total cell number was counted by trypan blue exclusion and the cell number of each subset was calculated by multiplying total cell number with percentage of each subset in flow cytometry analysis. IgG concentration was quantified by ELISA. (B) Histograms of BAFF-R expression in total population overlaid at various culture periods. Isotype controls were indicated as filled histograms. (C) Changes of BAFF-R and TACI expression in B cell subsets. The MFI value at day 0 represented that of freshly isolated GC-B cells. One representative data from four independent experiments is shown.

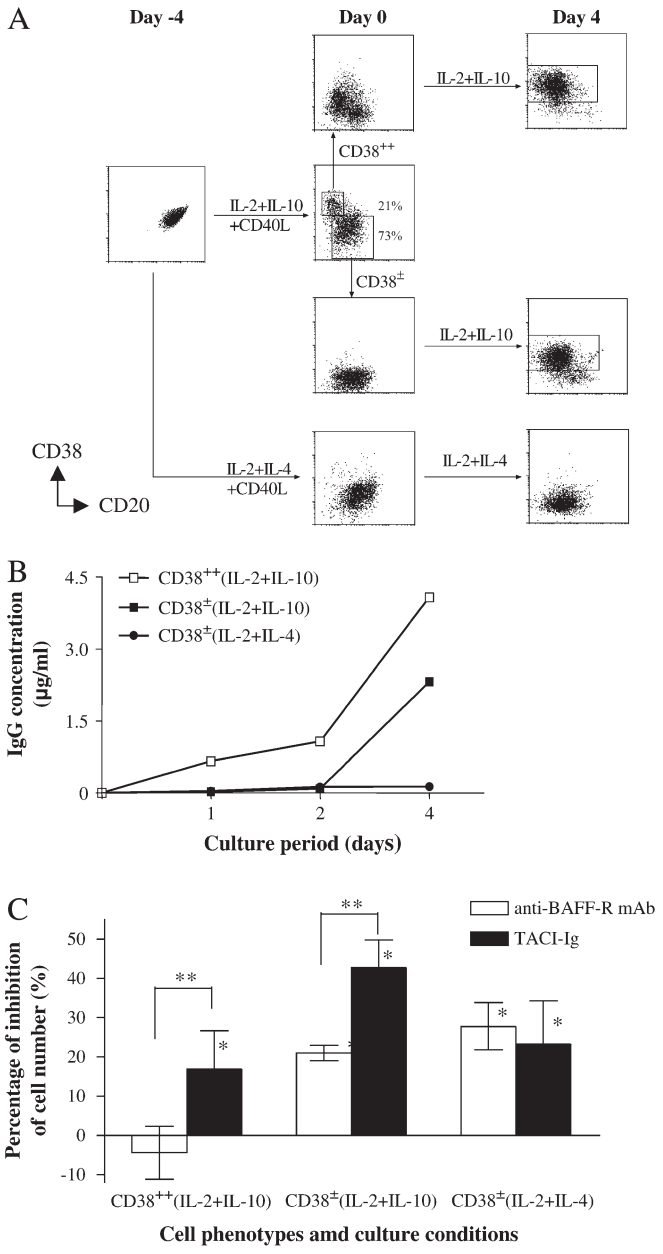


**Fig. 5.** BAFF plays an important role in GC-B cell differentiation to PCs via distinct receptors. $CD20^+CD38^+$ centroblasts were first cultured with IL-10 or IL-4 in the presence of IL-2, CD40L and HK cells for 4 days. $CD38^{\pm}$ and $CD38^{++}$ subsets were then separated from the cells cultured with IL-10 by MACS column and further cultured with IL-2, IL-10 and HK cells without CD40L for an additional 4 days. Simultaneously, the third batch of $CD38^{\pm}$ cells was cultured with of IL-4 in place of IL-10 throughout the first and secondary cultures. Each day in the secondary culture, surface marker changes and IgG secretion in the supernatants were examined. Anti-BAFF-R mAb or TACI-Ig (both 10 μg $ml^{-1}$) was added in the beginning of the secondary culture. (A) Phenotypic changes of B cell subsets in two-step cultures examined by CD20 and CD38 markers. (B) IgG secretion in each subset measured by ELISA. (C) BAFF-R blocking and BAFF depletion effect on each subset. The percent inhibitions of viable cell number were based on 100% of the control culture where isotype-matched control in place of specific blocking agent was added. Averages from four independent experiments were represented with error bars (standard deviation). Single asterisk denotes significant inhibition compared with the respective control ($P < 0.05$). Double asterisks denote significant difference between two inhibitions ($P < 0.05$).

amplified PCR products. This result was supported by flow cytometry staining for BAFF. All four primary FDCs showed small but significant positive staining for BAFF (Fig. 6B). The production of BAFF by FDC/HK cells was further confirmed by direct demonstration of BAFF in FDCs *in vivo* (Fig. 6C). The cytospin preparations of FDCs were stained with anti-BAFF mAb, control mAb or anti-DRC-1 (known as an FDC marker) and examined under a de-convolution microscope (39). To exclude false positive by contaminating cells, we examined BAFF expression in single FDCs, which were not clustered with B cells but could be identified by their size and abundant cytoplasm (40). Both anti-DRC-1 and anti-BAFF mAb co-stained FDCs. There was no green staining but only blue nuclear staining in samples stained with control mAb and DAPI.

## Discussion

A precise control of immune reaction is crucial to protect the body by eliminating invading pathogens, while preventing autoimmune responses. To this end, full activation of lymphocytes is controlled by various secondary signals in addition to those signals triggered by the antigen receptors. Moreover, some of those secondary signals have further fine-tuning mechanisms not only by changing stimulating ligand concentration but also by altering responsive receptors as demonstrated in the B7-CD28-CTLA-4 system of T cell activation (41). BAFF–BAFF-R system appears to be similarly modulated in generating PCs from GC-B cells.

Immunohistological staining and flow cytometry analysis of human tonsillar B cell subsets revealed that $IgD^+$ naive B cells in the mantle zone expressed the highest level of BAFF-R among B cell subsets. Since a specific mAb against BAFF-R completely neutralized the co-stimulatory activity of BAFF, BAFF-R appears to be the dominant receptor for naive B cells. In the early stage of GC-B cell activation, BAFF-R was up-regulated transiently. The addition of TACI-Ig or anti-BAFF-R inhibited the cellular proliferation of GC-B cells comparably, suggesting the dominant role of BAFF-R for GC-B cells. This conclusion is consistent with the *in vivo* data obtained from mice with defective BAFF-R (19, 20, 42). These mice did not respond to BAFF *in vivo* and revealed severe defects in GC reaction (43). However, the functional role of other receptors in GC response is not clearly understood. When GC-B cells differentiated into Ig-secreting PCs, TACI and BCMA were induced, while BAFF-R was down-regulated. Since the addition of TACI-Ig inhibited PC generation more than specific inhibition by anti-BAFF-R mAb, BAFF-R is no longer a dominant receptor and BAFF signaling is mediated through TACI or BCMA as GC-B cells differentiate to PCs. Indeed, the addition of anti-BAFF-R in Ig-secreting PC ($CD38^{++}$) culture exhibited no effect, while TACI-Ig inhibited. Our conclusion is further supported by recent reports that BCMA mediates BAFF signals in the generation of PCs from human splenic memory B cells *in vitro* and that the generation of the long-lived PCs is impaired in BCMA knockout mice (26, 30). Since PCs/PC precursors migrate out from the GC as they differentiate and BCMA and TACI can bind to another ligand APRIL, which we were not able to detect either in FDCs or GC-B cells in the GC (data not shown), the change in receptor usage may imply the

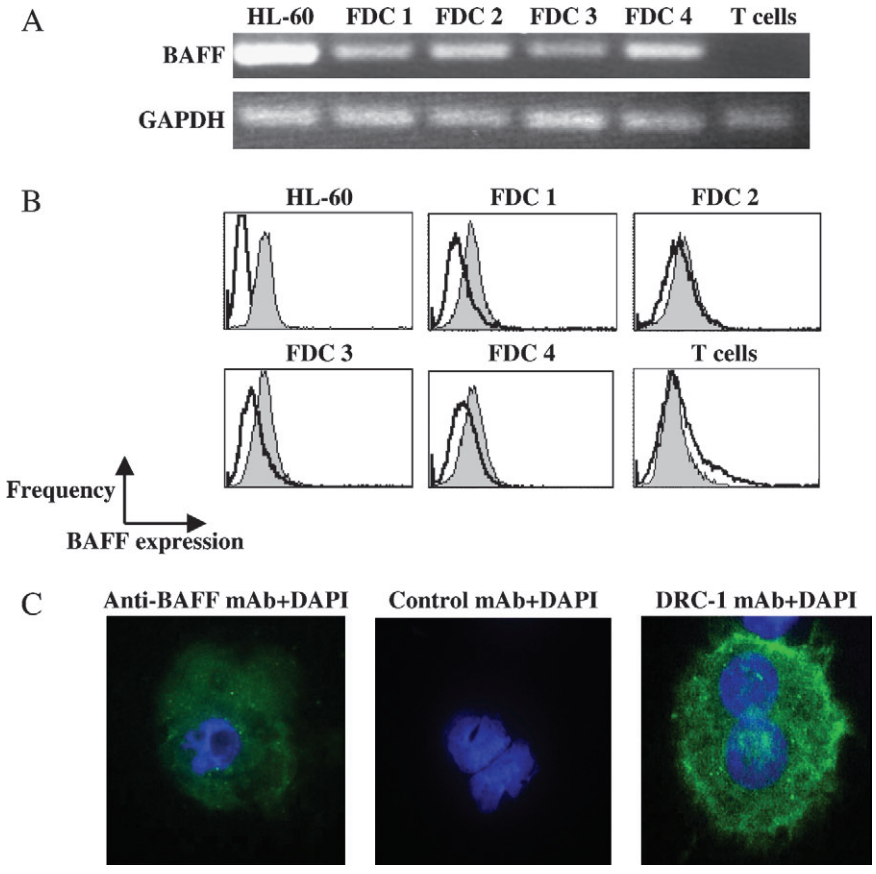


**Fig. 6.** FDCs produce BAFF. (A) BAFF mRNA expression in primary FDCs. Primary FDCs were isolated from four donors and RT–PCR reaction was performed as described in Methods. HL-60 cells and T cells were included to serve as a positive and negative control, respectively. (FDCs from one donor, being used *in vitro* experiment, were denoted as HK.) (B) BAFF expression on primary FDCs measured by flow cytometry. FDCs were incubated with anti-BAFF (filled histogram) or control mAbs (solid line) followed by FITC-conjugated goat anti-mouse-Ig antibody. (C) BAFF expression *in vivo*. Cytospin preparations of human FDC clusters were stained with anti-BAFF mAb (left), control mAb (middle) or FDC marker DRC-1 (right) followed by FITC-conjugated goat anti-mouse-Ig antibody (green). DAPI was used for nuclear counter-staining (blue). Original magnification ×630.

change in signaling ligand—BAFF to APRIL or both. Therefore, the final step of the humoral immune response is likely to be regulated by its microenvironment through exceptionally complex but precise mechanisms.

Our study has important clinical implications. The existence of long-lived PCs has been demonstrated in murine model of SLE and suggested in human (44). The long-lived PCs are generated in the GC (45). Analysis of these cells in humans, however, has proven difficult due to the extremely low number of their precursors in peripheral blood lymphocytes. Here, we demonstrate the putative precursors that are, BAFF-R$^{low}$, TACI$^{high}$ and BCMA$^{+}$ B cells. Since one of the reasons of the inability to cure autoimmune diseases is the lack of detailed knowledge of the PC populations involved in the particular disease (46), our study is significant to define these little known precursor cells in autoimmune diseases. The clinical implication of differential usage of BAFF-Rs is further demonstrated in malignant B cells. As normal counterparts, lymphoma and multiple myeloma cells express different BAFF-Rs that correspond to their differentiation stages (36, 47, 48). In some multiple myeloma cells, BAFF-R is down-regulated, while TACI and BCMA up-regulated like PCs generated from GC-B cells. These tumors produce BAFF, suggesting an important functional role of BAFF and its receptors for autocrine growth. Therefore, our studies suggest that TACI and BCMA may be a proper target for new putative therapeutic reagents.

The cellular source of BAFF in the GC has been controversial. Some reports suggest the expression of BAFF in GC-B cells, while others have not shown BAFF production in B cells (36, 38, 47, 48). Recently, it has been reported that radioresistant stromal cells are the major *in vivo* source of BAFF (49). Consistent with this finding, we were able to detect BAFF in primary FDCs by RT–PCR and flow cytometry. In addition, the presence of BAFF in FDCs *in vivo* is further confirmed by immunofluorescent staining experiments with *ex vivo* FDCs. Furthermore, the recovery of GC-B cells cultured with HK cells was significantly reduced by BAFF-depleting reagents, suggesting that FDC/HK produces functional BAFF.

In summary, we have analyzed the expression and function of BAFF-Rs in tonsillar B cells. Our data suggest that B cells receive BAFF signal through different receptors as they differentiate to PCs. Initially, naive B and GC-B cells use BAFF-R, whereas afterwards PC precursors and PCs use

BCMA and TACI. We also show that FDCs in GC produce BAFF. Further studies on mechanisms for expression and function of BAFF-Rs in the patients with autoimmune diseases and B cell lymphomas will provide important clues for a more efficacious treatment.

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**Abbreviations**

| | |
|---|---|
| APRIL | a proliferation-inducing ligand |
| BAFF | B cell-activating factor of the tumor necrosis factor family |
| BCMA | B cell maturation antigen |
| CD40L | CD40 ligand |
| DAPI | 4′,6-diamidino-2-phenylindole |
| DRC-1 | dendritic cell reticulum-1 |
| FDC | follicular dendritic cell |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| GC | germinal center |
| [$^3$H]TdR | [$^3$H]thymidine |
| MFI | mean fluorescent intensity |
| M-MLV RT | Moloney murine leukemia virus reverse transcriptase |
| PC | plasma cell |
| RT | reverse transcription |
| SLE | systemic lupus erythematosus |
| TACI | transmembrane activator and calcium modulator and cyclophilin ligand interactor |

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