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Title: A proliferation-inducing ligand sustains the proliferation of human naïve ($CD27^-$) B cells and mediates their differentiation into long-lived plasma cells *in vitro* via transmembrane activator and calcium modulator and cyclophilin ligand interactor and B-cell mature antigen

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Abstract: Long-lived plasma cells (PCs) contribute to humoral immunity through an undefined mechanism. Memory B cells, but not human naïve B cells, can be induced to differentiate into long-lived PCs in vitro. Because evidence links a proliferation-inducing ligand (APRIL), a tumor necrosis factor family member, to the ability of bone marrow to mediate long-term PC survival, we reasoned that APRIL influences the proliferation and differentiation of naïve B cells. We describe here the development of a simple cell culture system that allowed us to show that APRIL sustained the proliferation of naïve human B cells and induced them to differentiate into long-lived PCs. Blocking the transmembrane activator and calcium modulator and cyclophilin ligand interactor or B-cell mature antigen shows they were required for the differentiation of naïve B cells into long-lived PCs in vitro. Our in vitro culture system will reveal new insights into the biology of long-lived PCs.

Abbreviations

Ab, antibody; APRIL, a proliferation-inducing ligand; Ara-C, arabinosylcytosine; BAFF, B-cell-activating factor; BCMA, B-cell mature antigen; BCL6, B-cell lymphoma 6; CD40L, CD40 ligand; cDNA, complementary DNA; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GCBCs, germinal center B cells; Ig, immunoglobulin; MACS, magnetically activated cell sorting; ODN, oligodeoxynucleotide; PBS, phosphate-buffered saline; PBs, plasmablasts; PCs, plasma cells; PE, phycoerythrin; PI, propidium iodide; PAX5, paired box gene 5; RT-PCR, reverse transcriptase-polymerase chain reaction; SEM, standard error of the mean; TACI, transmembrane activator and calcium modulator and cyclophilin ligand interactor; TNF, tumor necrosis factor; XBP1, X-box binding protein 1

Abstract

Long-lived plasma cells (PCs) contribute to humoral immunity through an undefined mechanism. Memory B cells, but not human naïve B cells, can be induced to differentiate into long-lived PCs *in vitro*. Because evidence links a proliferation-inducing ligand (APRIL), a tumor necrosis factor family member, to the ability of bone marrow to mediate long-term PC survival, we reasoned that APRIL influences the proliferation and differentiation of naïve B cells. We describe here the development of a simple cell culture system that allowed us to show that APRIL sustained the proliferation of naïve human B cells and induced them to differentiate into long-lived PCs. Blocking the transmembrane activator and calcium modulator and cyclophilin ligand interactor or B-cell mature antigen shows they were required for the differentiation of naïve B cells into long-lived PCs *in vitro*. Our *in vitro* culture system will reveal new insights into the biology of long-lived PCs.

Key words: A proliferation-inducing ligand, Transmembrane activator and calcium modulator and cyclophilin ligand interactor, B-cell mature antigen, Long-lived plasma cell, Naïve B cell

1. Introduction

A proliferation-inducing ligand (APRIL) is a member of the tumor necrosis factor (TNF) family of cytokines that regulate the growth, maturation, differentiation, and survival of B cells [1–4]. APRIL signaling through its receptors, which are the members of the TNF receptor family, B-cell maturation antigen (BCMA), and transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), mediates the late stage of B cell development [5–11]. In a mouse model, APRIL deficiency decreases the ability of the bone marrow to support plasma cell (PC) survival [12]. APRIL is secreted by neutrophils, macrophages, and bone-marrow stromal cells *in vivo*, and lipopolysaccharide or interferon-γ induces dendritic cells to produce APRIL *in vitro*. These findings suggest that APRIL may be related to inflammation and innate immunity. Indeed, APRIL is implicated in autoimmune disorders, particularly systemic lupus erythematosus (SLE) and Sjogren's syndrome (SS). For example, the serum levels of APRIL in patients with SLE and SS are elevated compared with those of healthy controls [13–16].

APRIL plays an important role in the survival of PCs in mice, but its contributions to human B cell development are yet to be fully defined, particularly during PC differentiation. *In vitro* culture systems have been reported in which human B cells are activated to drive the PC phenotype [17,18]. For example, Ettinger et al. [19–21] reported that IL-21 could induce human naïve and memory B cells to acquire a PC phenotype following stimulation through BCR and CD40. Such PCs express the surface markers $IgD^{-}CD38^{++}$ and $BCMA^{+}$, although it is unclear if they are involved with CD138 (syndecan-1) expression, which is a specific marker for bone marrow-resident and terminally differentiated PCs [22]. Unmethylated CpG-oligodeoxynucleotides (ODNs) induce differentiation of memory B cells to PCs in the absence of BCR stimulation [23]. Huggins et al. [23] demonstrated that naïve B cells can differentiate into PCs after addition of a CpG-ODN, CD40L, IL-2, IL-6, IL-10, IL-15, and

hepatocyte growth factor (HGF) in a three-step culture system, and that the induced PCs express CD138. Cytokines such as IL-6, insulin-like growth factor-1, and HGF are secreted by bone-marrow stromal cells, and the local environment in the bone marrow is thought to support the survival of PCs [24]. Our group recently reported an immunosuppressive agent susceptibility for the differentiation of human B ($CD19^+$) cells *in vitro* that employs IL-21, CD40L, and CpG-ODNs. This system induces IgG production but does not maintain the survival of PCs for a long period [25]. Therefore, in the present study, we added APRIL to this *in vitro* culture system and show that APRIL supports the differentiation of naïve B cells into PCs, which efficiently secrete IgG and almost no changes in proliferation. Moreover, a receptor-blocking assay shows that APRIL via TACI and BCMA plays an important role in the differentiation of human naïve B cells to PCs *in vitro.*

2. Materials and methods

2.1 Isolation and culture of B cells

This study followed the Declaration of Helsinki, and all subjects provided informed consent for participation. Peripheral blood mononuclear cells were isolated using a Ficoll–Hypaque density gradient (Sigma-Aldrich, St Louis, MO, USA) from 10 healthy donors (ages 21–63 years; average, 37.6 ± 13.9 years; seven males). To isolate memory B cells from naïve B cells, mononuclear cells were subjected to negative selection using a B-cell isolation kit (Miltenyi Biotech, Auburn, CA, USA). The unlabeled B cells were passed through the column, washed with buffer, and subjected to magnetic-activated cell sorting (MACS) using CD27-conjugated microbeads according to the manufacturer's instructions.

The cells were cultured in basal B-cell culture medium composed of Iscove's modified Dulbecco's medium (Sigma–Aldrich) containing 10% fetal calf serum (FCS; Thermo Scientific

HyClone, NYSE, TMO) supplemented with 50 µg/ml human transferrin–selenium and 5 µg/ml human insulin (Gibco Invitrogen Co., Carlsbad, CA, USA) in the presence of the molecules as follows: 50 ng/ml IL-21, 2.5 µg/ml phosphorothioate CpG-ODN 2006, 50 ng/ml histidine-tagged soluble recombinant human CD40L, and 5 µg/ml anti-polyhistidine monoclonal antibody (mAb). We added TNF family members to the naïve ($CD27^-$) B-cell culture medium as follows: 500 ng/ml recombinant human APRIL or 50 ng/ml recombinant human B-cell-activating factor (BAFF). We used an anti-human IgM affinity-purified goat polyclonal Ab (4 µg/ml) to stimulate the BCR. Purified naïve ($CD27^-$) B cells were added to 96-well flat-bottom plates (1.6×10^4 cells per well).

2.2 Reagents

For cell culture, recombinant human IL-21 was purchased from Miltenyi Biotech. Recombinant human APRIL, recombinant human BAFF, histidine-tagged soluble recombinant human CD40 ligand, and an antipolyhistidine mAb were from R&D Systems (Minneapolis, MN, USA). Phosphorothioate CpG-ODN 2006 was purchased from Invivogen (San Diego, CA, USA) and Ara-C (cytosine arabinoside) from Sigma–Aldrich.

For BCR stimulation, anti-human IgM affinity purified goat polyclonal Ab was purchased from R&D Systems (Minneapolis, MN, USA).

2.3 Receptor-blocking assay reagents

Anti-human APRIL (10 µg/ml), 10 µg/ml anti-human BCMA (10 µg/ml), and anti-human TACI monoclonal Abs (mAbs; 10 µg/ml) were purchased from R&D Systems (Minneapolis,

MN, USA). Functional-grade mouse IgG1κ isotype and rat IgG2aκ isotype controls were purchased from eBioscience.

2.4 Flow cytometric analysis

To determine differentiation stages, the cells were reacted with fluorescein isothiocyanate (FITC)-anti-CD38 (eBioscience), PE-anti-138, and APC-anti-CD19 mAbs (BD Biosciences, San Jose, CA, USA). Dead cells were stained with propidium iodide (PI; Sigma-Aldrich). To measure the expression levels of IgG on the surface of naïve ($CD27^-$) B cells, cells were reacted with FITC-anti-IgG (eBioscience). FITC-BCMA/TNFRSF17 antibodies from Novus Biologicals (Littleton, Colorado, USA) and APC-anti-hTACI/TNFRSF13B antibodies (R&D Systems) were used to measure APRIL receptor expression on the surface of naïve ($CD27^-$) B cells. All flow cytometric analyses were performed using a FACS Calibur dual-laser flow cytometer using Cell Quest acquisition analysis software (BD Biosciences).

2.5 Enzyme-linked immunosorbent assay

Ninety-six-well plates were coated with a polyclonal $F(ab)_2$ goat anti-human IgG heavy chain Ab (Biosource, Camarillo, CA, USA) or affinipure $F(ab')_2$ goat anti-human IgM Fc5μ fragment specific Ab (Jackson Immunoresearch Laboratories, West Grove, PA, USA) at 4°C overnight.

The plates were washed five times with phosphate-buffered saline (PBS; Sigma-Aldrich) containing 0.1% Tween 20. A 100 μl aliquot each of the supernatant from each culture step, media alone, and IgG standards (to generate the calibration curve) were diluted 1:200 to

1:10,000 and IgM standards (to generate the calibration curve) were diluted from 1:1600 to 1:80,000 with PBS and incubated in the wells over night at 4°C.

The plates were then washed five times with PBS containing 0.1% Tween 20 and incubated with horseradish peroxidase-conjugated anti-human IgG or horseradish peroxidase-conjugated anti-human IgM (Jackson Immunoresearch Laboratories, West Grove, PA, USA) for 2 h at room temperature and then washed five times with PBS containing 0.1% Tween 20.

After the addition of TMB substrate (Thermo Scientific, Rockford, IL, USA), the optical density was measured at 630 nm using a PowerScan4 Microplate Reader (DS Pharma Medical Co.), and total IgG or IgM levels were determined from the calibration curve.

2.6 BrdU cell proliferation assay

BrdU incorporation by naïve ($CD27^-$) B-cell-derived PCs was quantified using a BrdU cell proliferation assay kit (Millipore, Billerica, MA, USA). Naïve ($CD27^-$) B cells were cultured for 10, 14, 21, 28, and 56 days, and $CD138^+$ PCs were collected at each time. The BrdU enzyme-linked immunosorbent assay (ELISA) assay was performed according to the manufacturer's instructions. The cells were pulsed with 20 µl of BrdU diluted 1:500 during the fifth day of culture. After adding BrdU, plates were centrifuged and cells were denatured with FixDenat solution and then incubated for 1 h at room temperature with 100 µl per well of diluted mouse anti-BrdU mAb and washed three times with wash buffer diluted 1:50. After addition of 100 µl per well of goat antimouse IgG Ab-peroxidase conjugate diluted 1:20,000 followed by incubation for 30 min at room temperature, the plates were washed three times. After removing the Ab conjugate, 100 µl per well of TMB peroxidase substrate was added and incubated for 30 min at room temperature in the dark. The absorbance was measured at 630 nm using a PowerScan4 Microplate Reader (DS Pharma Medical Co.). The blank corresponded to

100 µl per well of culture medium with BrdU, and the background corresponded to 100 µl per well of cells plated in culture medium without BrdU.

2.7 Quantitative real-time polymerase chain reaction

An miRNeasy Mini Kit (QIAGEN) was used to extract RNA from cultured naïve ($CD27^-$) B cells on days 0, 7, 14, 21, 28, and 35. The RNAs served as templates for cDNA synthesis using a high-capacity cDNA synthesis kit (Applied Biosystems, CA, USA). The cDNA samples were amplified using a SYBR Premix Ex Taq Kit (Takara Bio, Inc., Shiga, Japan). The primer sequences and conditions used for quantitative real-time polymerase chain reaction (qRT-PCR) are detailed in Supplementary Methods (1). A real-time reverse transcription-PCR (RT-PCR) was performed using an MX3000P (Agilent Technologies, Loveland, CO). Relative transcript levels were calculated using the comparative CT method [26]. Gene expression levels were normalized to those of the housekeeping gene *GAPDH*.

2.8 IgH chain V region mutation analysis

Total RNA was isolated from naïve ($CD27^-$) B cells on days 0 and 28, from memory ($CD27^+$) B cells on day 0, and cDNA was synthesized using random primers. Single-strand cDNA synthesis was performed using a high capacity cDNA synthesis kit (Applied Biosystems, CA, USA). IgH chain V (*IGHV*) regions were amplified from cDNA using PrimeSTAR GXL DNA Polymerase (Takara Bio, Inc., Shiga, Japan). *VH-CH* transcripts were amplified using the *VH* forward primer and the CHµ or CHγ reverse primer. The primer sequences and conditions used for qRT-PCR are detailed in Supplementary Methods (2). Amplicons were gel purified, and poly(dA) was added to their 3′ ends using the Mighty TA-cloning Reagent Set for PrimeSTAR (Takara Bio, Inc.) and were cloned using a pGEM-T Easy TA Cloning Kit

(Promega). PCR products were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and analyzed using an Applied Biosystems Prism 310 Genetic Analyzer.

2.9 Statistical analysis

The data are expressed as median values and ranges. The significance of differences between variables was evaluated using a paired or unpaired Student *t* test with SPSS Statistics 20 software (IBM Corporation, Armonk, NY). A two-tailed *P* value < 0.05 was considered statistically significant.

3. Results

3.1 Absence of IGHV somatic hypermutation in pooled CD27$^-$ B cells collected from healthy human donors

We separated naïve (CD27$^-$) B-cell-enriched populations from total peripheral blood B cells using negative selection. These cells did not show patterns of *IGHV* somatic hypermutation similar to those of classical memory B cells. In contrast, memory (CD27$^+$) B cells showed patterns of *IGHV* somatic hypermutation (Fig. 1).

3.2 APRIL supports longer survival of naïve (CD27$^-$) B-cell-derived PCs in vitro and increases IgG secretion compared with BAFF

We compared the effects of BAFF and APRIL on naïve (CD27$^-$) B cells *in vitro*. In the presence of exogenous BAFF, there were significant differences in the differentiation rate of PCs (35.6 ± 5.2 % vs 4.2 ± 2.7 %) on day 14 and (21.3 ± 5.9 % vs 2.2 ± 1.8 %) on day 25 between cells treated with BAFF and the controls (P<0.01, P<0.01, respectively; Fig. 2A) ,but there were no significant differences (3.3 ± 2.1 % vs 2.0 ± 0.4 %) on day 7, (4.3 ± 2.9 % vs 2.2

± 1.8 %) on day 35 (Fig. 2A). IgG concentrations increased significantly in supernatants of cultures treated with BAFF compared with those of controls on day 14 ($P < 0.01$), on day 25 ($P<0.05$), on day 35 ($P<0.05$) (Fig. 2B). APRIL increased the differentiation rate, and there were significant differences (24.7 ± 9% vs 1.3 ± 2.8%) on day 25 and (19.6 ± 4.6% vs 2.2 ± 1.8 %) on day 35 between cells treated with APRIL and the controls ($P < 0.01$, $P< 0.01$, respectively; Fig. 2A). Moreover, the production of IgG by cells treated with APRIL was significantly higher compared with controls on days 25 and 35 ($P < 0.05$, $P < 0.001$, respectively; Fig. 2B).

3.3 APRIL promotes the differentiation of naïve ($CD27^-$) B cells derived from B-cell-derived plasmablasts into mature PCs

APRIL maintained the number of naïve ($CD27^-$) B-cell-derived PCs on days 21 ($P < 0.05$) and 28 ($P < 0.01$) compared with the controls (Fig. 3A). To ensure that the progeny PCs were derived from naïve ($CD27^-$) B cells on day 28, mRNA was isolated from total B cells on day 28, and analysis of *IGHV* mutations was performed. *IGHV* somatic hypermutation was not detected (Fig. 3B).

We next determined the effect of APRIL on naïve ($CD27^-$) B cells during each stage of differentiation. Fluorescence-activated cell sorting (FACS) analysis shows that the plasmablast (PB) differentiation rate (56.6 ± 3.3 %) of controls was significantly increased compared with cells treated with APRIL (31.9 ± 1.7 %) on day 25 ($P < 0.001$; Fig. 3C). However, we did not detect the differentiation of PCs in control cultures compared with cultures treated with APRIL on day 25 (Fig. 2A). In addition, we estimated the effect of APRIL on more mature PCs. When naïve ($CD27^-$) B cells were treated with an anti-human APRIL mAb on day 21, there weresignificant differences on the rate of differentiation of naïve ($CD27^-$) B-cell derived PCs between Ab-treated cells (75.9 ± 7.6 %) and controls (72.9 ± 4.4 %) on day 35 (Fig. 3D).

To determine why naïve ($CD27^-$) B cell appeared to be differentiated into more mature PCs, we gated on PCs on days 14, 21, 28, and 56 and then evaluated cell-surface expression of CD138 on naïve ($CD27^-$) B-cell-derived PCs compared with that of PBs. We found that the PCs expressed significantly higher levels of CD138 compared with those of PBs, and the expression of CD138 on the PC surface was increased in the presence of APRIL (Fig. 3E).

3.4. Naïve ($CD27^-$) B-cell class-switching and induction of terminal differentiation by APRIL

Next, we determined the stage of differentiation when naïve ($CD27^-$) B cells underwent Ig class switching. All naïve ($CD27^-$) B cells were stained with FITC-anti IgG on days 1, 4, 7, and 14. The upregulation of IgG on the surface of naïve ($CD27^-$) B cells in the presence or absence of exogenous APRIL was detected on days 1,4, and 7 (all $P > 0.5$). In contrast, IgG levels increased in the presence of APRIL compared with those of control cells on day 14 ($P < 0.01$; Fig. 4A).

We next investigated the effect of stimulating the BCR on the differentiation of naïve ($CD27^-$) B cells. Stimulating the BCR significantly promoted Ig class switching of naïve ($CD27^-$) B cells on days 1, 4, 7, and 14 compared with the controls ($P < 0.01$, $P < 0.001$, $P < 0.001$ and $P < 0.0001$, respectively; Fig. 4A). Moreover, naïve ($CD27^-$) B cells were cultured with the APRIL addition in the presence or absence of BCR stimulation. FACS analysis revealed significant differences in the percentages of PBs between BCR-stimulated (day 7, 92.5 ± 2.2%; day 14, 86.7 ± 3.4 %; day 25, 60.1 ± 5.5 %) and BCR-unstimulated cells (day 7, 12.8 ± 1.2%; day 14, 26.0 ± 5.9 %; day 25,31.8 ± 2.1 %) on days 7, 14, and 25 ($P < 0.0001$, $P < 0.0001$, $P < 0.0001$, respectively; Fig.4B). In contrast, BCR stimulation did not promote the differentiation of naïve ($CD27^-$) B cells into PCs compared with BCR-unstimulated on days 7, 14, and 25 (Fig. 4B). Further, IgG levels in culture supernatants of BCR-stimulated cells were significantly higher compared with those on day 14 ($P < 0.05$), although there were no

significant differences between BCR-stimulated and BCR-unstimulated cells on day 25 (Fig. 4C).

Next, we assessed the ability of naïve ($CD27^-$) B-cell-derived PCs to synthesize DNA to determine whether naïve ($CD27^-$) B-cell-derived PCs were terminally differentiated by using a BrdU ELISA assay. We found a decreasing linear rate of absorbance per PC count from day 10 that changed little between days 21 and 56 (Fig. 5A), suggesting that naïve ($CD27^-$) B-cell-derived PCs lost the ability to synthesize DNA up to day 21. When we added Ara-C to the culture medium on day 21 and used FACS to analyze cell proliferation and survival on days 28 and 56, we found that only PCs treated with Ara-C survived on days 28 and 56. In contrast, PBs and activated B cells did not survive in the presence of Ara-C on day 28 (Fig. 5B). The numbers of PBs in the control cultures on day 28 were increased significantly compared with cultures treated with Ara-C (P <0.0001), but there were no significant differences on the numbers of PCs (Fig. 5C), and the IgG levels in control culture supernatants were increased significantly compared with cultures treated with Ara-C on day 28 (Fig.5D; P< 0.01). On the other hand, the Ara-C addition significantly decreased the numbers of PCs on day 21 to compared with the control group when it was administered on day 14 (P<0.05; Fig.5C).

We next compared IgG production per naïve ($CD27^-$) B-cell-derived PCs and per day in untreated cultures treated with those treated with Ara-C. To calculate the rate of IgG production per naïve ($CD27^-$) B-cell-derived PC and per day, we replenished the culture medium and cultured the cells for 1 day. The values were calculated by dividing the amount of IgG in the culture supernatant by the number of viable PCs at the time of collection of the culture supernatant. We found that when Ara-C was added on day 21, there were no detectable differences in IgG levels on days 28 and 56 compared with the controls (Fig. 5E). When we replenished the culture medium and analyzed the culture supernatant by ELISA, these cultured cells were analyzed by FACS on day 7, 14, 21 and when we added Ara-C to the culture medium, we collected all these cells to analyze them, the details of which are shown in Supplementary

scheme (1).

3.5. APRIL-induced terminal differentiation of naïve B-cell-derived PCs correlates with increased levels of PRDM1 and XBP1 mRNAs

When we examined the gene expression profile of naïve ($CD27^-$) B cells, we found that the levels of *PAX5* and *BCL6* mRNAs decreased (Fig. 6A, B), while those of *PRDM1* and *XBP1* increased (Fig. 6C, D).

3.6 APRIL induces the differentiation of naïve B cells to PCs via BCMA and TACI

Using FACS, we estimated the levels of BCMA and TACI expressed by naïve ($CD27^-$) B cells on days 7, 14, and 21 and found that BCMA levels increased from days 7 to 14 in the absence of exogenous APRIL with little change on day 21. TACI levels increased from days 7 to 14 in the absence of exogenous APRIL and then returned to their initial levels on day 21 when germinal center B cells (GCBCs) differentiated into PCs (Fig. 7A).

When we used a neutralizing Ab to block APRIL binding, BCMA had no detectable effect on the rate of proliferations of naïve ($CD27^-$) B-cell-derived PCs on day 25 when the Ab was administered on days 1 and 7 (Fig. 7B). However, administration of antibodies against either BCMA or APRIL on day 14 inhibited differentiation into PCs on day 25 (Fig. 7C). IgG and IgM levels in culture supernatants were significantly decreased compared with those of controls on day 25 when the Ab was administered on day 14 ($P < 0.05$, $P < 0.05$, respectively; Fig. 7D). FACS analysis revealed significant differences in the percentages of PBs on day 25 between antibodies against BCMA-treated on day 14 and control cells (92.6 % +/- 1.5 % vs 31.8 % +/- 2.1%; $P < 0.0001$), but naïve ($CD27^-$) B cells could not differentiate into PCs (5.2 +/- 0.6%) to compare with control cells on day 25 (31.9 ± 1.7 %) (Fig. 7E).

Moreover, when a neutralizing Ab against TACI was added to cultures on day 14, PC counts on day 25 were unchanged (Fig. 7B, C). When TACI-neutralizing antibodies were added to

cultures on day 14, IgM production was increased significantly (P <0.0001), and IgG production was decreased significantly (P <0.05) compared with controls on day 25 (Fig. 7D).

4. Discussion

Human memory B cells differentiate into long-lived PCs *in vitro* [24]. In contrast, Huggins et al. [23] differentiated human naïve ($CD27^-$) B cells into $CD138^+$ PCs but did not show that these cells differentiated into long-lived PCs *in vitro*. Therefore, we attempted to induce human naïve ($CD27^-$) B cells to differentiate into long-lived PCs *in vitro*. Peripheral blood B cells consist of naïve and memory B cells, and they can be isolated because the latter express CD27 [27, 28]. However, memory B cells may not express $CD27^-$, and their patterns of *IGHV* somatic hypermutation are similar to those of classical $CD27^+$ memory B cells [28]. In the present study, we did not detect *IGHV* somatic hypermutations in peripheral blood cells enriched for naïve ($CD27^-$) B cells on day 0, unlike memory ($CD27^+$) B cells. Further, after culture for 28 days, we found that the PCs on day 28 were indeed derived from naïve ($CD27^-$) B cells, because the surviving B cells did not express mutated rearranged *IG* genes. Therefore, we suggest that selection based on the lack of detection of CD27 was sufficient to ensure that the differentiation of naïve B cells could be monitored using analysis of *IGHV* mutations.

We examined the effects of the TNF family members BAFF and APRIL in our culture system. The receptors that bind to APRIL, BAFF, or both include the BAFF receptor (BAFF-R), TACI, and BCMA. Mature B cells express BAFF-R, which binds BAFF but not APRIL. In contrast, TACI and BCMA bind BAFF and APRIL. TACI is expressed at relatively high levels by GCBCs, whereas BCMA is expressed mainly by PCs. APRIL and BAFF bind to TACI with equal affinity, and APRIL binds BCMA with higher affinity than BAFF [28–31]. APRIL is a ligand of BCMA and TACI, with TACI expressed strongly by GCBCs and BCMA expressed mostly by PCs. The biological affinity of TACI for APRIL and BAFF is equal, whereas BCMA

has a higher affinity for APRIL than for BAFF [32-35]. Bone marrow PCs from *Bcm*-knockout mice have a lower survival rate compared with those from wild-type mice, indicating that signal transduction through BCMA is related to the long-term survival of PCs [3,11,36]. The pattern of temporal expression of BAFF suggests that it acts during earlier stages of B-cell differentiation than APRIL. For example, when mouse B cells are cultured on BAFF-feeder cells, they differentiate into memory B-like cells. However, these cells must be transferred into mice to differentiate into long-lived PCs [37].

In our study, addition of BAFF promoted naïve ($CD27^-$) B cells differentiation into immature PCs that did not exhibit long-term survival; however we did not confirm these cells further differentiation into more mature PCs *in vitro* compared with cells treated with APRIL.

Here, we show the addition of recombinant APRIL to the medium, maintained cultures of naïve ($CD27^-$) B-cell-derived PCs for 56 days. First, we demonstrated upregulated expression of the surface antigen CD138 and increased secretion of IgG by the B cells as differentiation progressed. This is consistent with evidence showing that CD138 expression increases gradually as PCs mature [24, 38, 39]. Additionally, we observed the effect of APRIL on each differentiation stage of naïve ($CD27^-$) B cell *in vitro*. With respect to IgG class switching, naïve ($CD27^-$) B-cell-derived PCs usually secreted IgM [40]. Therefore, we estimated how APRIL influenced switching from IgM to IgG by naïve ($CD27^-$) B cells in our culture system. We detected increased expression of cell-surface IgG by naïve ($CD27^-$) B cells in cultures treated with APRIL or not, indicating the switch from IgM to IgG production on days 1, 4, and 7. It has been already reported that IL-21, CD40L and CpG-ODN, which were added to the B-cell culture medium, induce IgG class switching [41-43]. Therefore, we expected that any of these cytokines would support class switching by naïve ($CD27^-$) B cells on days 1, 4, and 7. However, APRIL significantly promoted naïve ($CD27^-$) class switching as compared with the

control groups at later culture time points. From the above, APRIL supported naïve ($CD27^-$) B cell IgG class switching and differentiation into a more mature PC phenotype [43, 44].

When we analyzed the expression of mRNAs specifically expressed by PCs, we found that the levels of *BCL6* and *PAX5* mRNAs were downregulated and those of *PRDM1* and *XBP1* were upregulated [16,21,23,45-52]. These findings support the conclusion that APRIL induced the differentiation of naïve ($CD27^-$) B cell into PCs.

However, the longevity of naïve ($CD27^-$) B-cell-derived PCs and calling them long-lived PCs are still questionable, because only indirect evidence is available on the longevity of PCs [53, 54]; that is, the differences in survival of PCs have important implications for sustaining host immunity. In the absence of exogenous APRIL, naïve ($CD27^-$) B-cell-derived PCs survived for approximately 14 days in our cell culture system. Therefore, it is possible that naïve ($CD27^-$) B cells differentiated into PCs after day 14, persisted until day 28 and then died rapidly. Therefore, the fate of PCs that were treated with Ara-C on day 21 must be addressed, because long-lived PCs survived as postmitotic cells [55-57]. Thus, we cultured naïve ($CD27^-$) B cells in the presence of the proliferation inhibitor Ara-C, which is used as an anticancer drug that inhibits cell proliferation by inducing DNA damage and apoptosis [58]. We found that only viable postmitotic PCs were present on days 28 and 56, and that there were no significant differences in IgG production of individual PCs between days 28 and 56.
From the above, naive ($CD27^-$) B cell-derived PC lost the ability to synthesize DNA by day 21 in the presence of APRIL, and IgG production of individual postmitotic PCs was completed by day 28.

The culture method described here is extremely simple, because it only involves the addition of APRIL, IL-21, CD40 ligands, and CpG-ODN and does not require unknown cytokines or interactions between B cells and stromal cells despite reports that together, APRIL, IL-6, and other factors maintain the viability of bone marrow-derived PCs or PCs generated *in vitro*, including PCs derived from memory B cells [29, 30]. Further, memory B cells differentiate into long-lived PCs when cultured in the presence of supernatants from the bone marrow-derived stromal cell line M2-10B4 [24], which supports long-term culture of human bone marrow stem cells. How M2-10B4 cells contribute to the survival of PCs is unknown, but memory B cells may require well-balanced support from stromal cells [31,59-61].

Cocco et al. [24] differentiated memory B cells into long-lived PCs by stimulating the BCR. Therefore, we investigated the effect of BCR stimulation on the proliferation and differentiation of naïve ($CD27^-$) B cells *in vitro.* We showed here that BCR stimulation induced naïve ($CD27^-$) B cells to undergo IgG class switching and to proliferate and differentiate into PBs but did not support differentiation of these cells into PCs *in vitro* (Supplementary Figure. 1). In addition, APRIL could support differentiation of naïve ($CD27^-$) B cells into PCs under normal circumstances; however, it was not affected by the addition of BCR stimulation. BCR stimulation promoted naïve ($CD27^-$) B cell proliferation; therefore, we assumed that APRIL was exhausted before these cells differentiated into PCs *in vitro*(Fig. 4B).
From the above mentioned, our study suggested that BCR stimulation was effective for naïve ($CD27^-$) B cells proliferation and class switching; however, we did not confirm that it affects the growth and survival of PCs *in vitro*, as it has been reported that BCR stimulation affects B cell selection or growth before B cells migrate to the bone marrow *in vivo* [62-64].

We show further that neutralizing antibodies against the APRIL receptor BCMA, which was upregulated on day 14 (Fig. 6D), influenced the differentiation of naïve ($CD27^-$) B cells into PCs on day 25 (Fig 7F). Moreover, when the anti-human APRIL mAb was administered on

day 21, it did not affect the survival of PCs on day 35. These results suggest that through BCMA, APRIL is required for the differentiation of PBs into more matured PCs but these PCs could survive without the APRIL addition after acquiring long-lived phenotype.

We found as well that the number of naïve ($CD27^-$) B-cell-derived PCs on day 25 was not altered by the addition of a neutralizing Ab against TACI despite the upregulation of TACI on day 14. However, IgM production on day 25 was increased significantly when cells were incubated with a neutralizing Ab against TACI on day 14 compared with controls, and that the production of IgG subclasses was suppressed on day 25. These findings are consistent with the requirement for TACI for IgG production by B-cell cultures derived from *Taci*-knockout mice [65-67]. In that splenic B-cell culture system, APRIL or BAFF cooperates with Toll-like receptor-9 agonists to induce B-cell differentiation, which is blocked by a neutralizing Ab against TACI [35].

5. Conclusion

Our results lead us to conclude that APRIL plays an important role in human naïve ($CD27^-$) B cells class switching through TACI and the differentiation of human naïve ($CD27^-$) B-cell derived PBs into more mature PCs through BCMA *in vitro*.
Additionally, naïve B ($CD27^-$) cells could survive without the APRIL supplementation *in vitro* after acquiring long-lived phenotype.

Y.M. designed the research, performed the experiments, and wrote the paper; H.M. and T.K. provided technical assistance and excellent advice; T.K. and K.K. designed the research.

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Conflict of Interest Disclosure

The authors declare no conflict of interest

Supplementary methods

(1) qPCR

Each well contained 20 μl of reaction mixture with cDNA, and the assay was performed as follows: incubation for 10 s at 95°C for initial denaturation, 40 cycles for 15 s at 95°C, 30 s at 60°C, 1 min at 95°C; 30 s at 60°C, and then gradual heating to 95°C to determine the dissociation curve.

The PCR primers were as follows:

PAX5; [5′-CGACATCTCCAGGCAGCTTCGG-3′ (forward) and
5′-TGTGGCGACCTTTGGTTTGGATCC-3′ (reverse)]
IRF4; [5′-GCCCAGCAGGTTCACAACTA-3′ (forward) and
5′-AGGTGGGGCACAAGCATAAA-3′ (reverse)]
PRDM1; [5′-GCGGGGAGAATGTGGACTGGGTA-3′ (forward) and
5′-CCCTCTGCCAATCCCTGAAACCTCA-3′ (reverse)]
XBP1; [5′-TCTGCTGAGTCCGCAGCAGGT-3′ (forward) and
5′-GGCTGGCAGGCTCTGGGGA-3′ (reverse)]
BCL6; [5′- TGAAATCTGTGGCACCCGTT-3′ (forward) and
5′-GCACCTTGGTGTTGGTGATG-3′ (reverse)]

(2) qPCR

Each well contained 20 μl of reaction mixture with cDNA, and the assay was performed as follows: 10 s at 98°C for initial denaturation, 30 cycles for 15 s at 55°C, and 30 s at 68°C. In addition, 12.5 μl of SYBR Premix Ex Taq (Takara Bio, Inc., Shiga, Japan) was added to the PCR products, and real-time PCR was performed under 10 minat 72°C. The PCR primers investigated in this study are as follows:

V5/6, [5′-C/GAA/GGTA/GCAGCTGC/G/TA/TGCAGTCA/TG-3′ (forward)]

CHμ, [5′-TCACAGGAGACGAGGGGGAA-3′ (reverse)]

CHγ, [5′-GAGTTCCACGACACCGTCAC-3′ (reverse)]

Supplementary scheme

(1)

When we replenished the culture medium and analyzed the culture supernatant by ELISA, these cultured cells were analyzed by FACS on day 7, 14, 21, and when we added Ara-C to the culture medium, we collected all these cells to analyze them, the details of which are shown.

Supplementary Figure

(A) Analysis of Ig class switching to estimate the effect of B-cell receptor (BCR) stimulation on the differentiation of naïve ($CD27^-$) B cells. Naïve ($CD27^-$) B cells were cultured and stained with a fluorescein isothiocyanate-conjugated anti-IgG antibody on days 1, 4, 7, and 14. We estimated cell-surface IgG levels of naïve ($CD27^-$) B cells cultured with BCR stimulation. These results indicate that BCR stimulation exerted a significant effect on naïve ($CD27^-$) B-cell class switching. Histograms showing expression of IgG determined on days 1, 4, 7, and 14 (median value; mean ± SED. **(B)** Fluorescence-activated cell sorting analysis revealed that BCR stimulation promoted the differentiation of naïve ($CD27^-$) B cells into PBs; however, it did not confirm that these cells were further differentiated into PCs.

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Figure Legends

Figure 1. Analysis of mutations in sequences encoding the IGHV. Total RNA was isolated from naïve ($CD27^-$) and memory ($CD27^+$) B cells on day 0. *VH5/6* rearrangements in memory ($CD27^+$) B cell **(A)** and naïve ($CD27^-$) B cell **(B)** subsets. **(A)** *VH5/6* was rearranged in cells preparations enriched in memory ($CD27^+$) B cells **(B)**. Somatic hypermutation of the *IGHV* region of *VH5/6* was not detected in preparations enriched in naïve ($CD27^-$) B cells. *IGHV* rearrangement junctions are indicated with a solid triangle. Naïve ($CD27^-$) and memory ($CD27^+$) B cells were collected from five independent healthy donors, and the data represent five independent experiments.

Figure 2. The effects of BAFF and APRIL on the differentiation of naïve ($CD27^-$) B cells. **(A)** Naïve ($CD27^-$) B cells were cultured and stained with fluorescein isothiocyanate-anti-CD38, phycoerythrin-anti-CD138, and APC-anti-CD19 mAbs on days 7, 14, 25 and 35. Fluorescence-activated cell sorting analysis of the expression of CD19, CD38, and CD138 *in vitro* by naïve ($CD27^-$) B cells isolated from the same donor. PCs were identified as $CD19^+CD38^{++}CD138^+$. **(B)** IgG production in the culture supernatants of cells treated or not with APRIL or BAFF on days 7, 14, 25, and 35. Naïve ($CD27^-$) B cells were collected from 10 healthy donors, and the data represent 10 independent experiments. The graphs show the mean ± standard error of the mean (SED). The statistical significance of the differences was evaluated using a paired *t* test. Significant changes from baseline are indicated with an asterisk ($*P < 0.05$, $**P < 0.01$).

Figure 3. The effect of APRIL on the differentiation of naïve B cells. **(A)** PC counts of cultures treated or not with APRIL. **(B)** To ensure that all PCs were derived from naïve ($CD27^-$) B cells, on day 28 we did not detect somatic *IGHV* hypermutation of *VH5/6*. *IGHV* rearrangement

junctions are indicated with a solid triangle. **(C)** Fluorescence-activated cell sorting analysis of the expression of CD19, CD38, and CD138 *in vitro* by naïve ($CD27^-$) B cells isolated from the same donor. PBs were identified as $CD19^+CD38^{++}CD138^-$, activated B cells as $CD19^+CD38^+CD138^-$. **(D)** Naïve ($CD27^-$) B cells were cultured in the presence of APRIL for 21 days and then cultured with the addition of APRIL for additional 14 days in the presence or absence of an anti-APRIL antibody and all these cells were analyzed by FACS on day 35. **(E)** We estimated CD138 levels on the surface of naïve ($CD27^-$) B-cell-derived PBs on day 14 and those of naïve ($CD27^-$) B-cell-derived PCs on days 14, 21, 28, and 56. CD138 expression by PCs was significantly increased. Histograms show CD138 expression monitored on days 14, 21, 28, and 56 (median value; mean ± SED). Naïve ($CD27^-$) B cells were collected from five healthy donors, and the data represent five independent experiments.

Figure 4. Analysis of Ig class switching to estimate the effect of B-cell receptor (BCR) stimulation on the differentiation of naïve ($CD27^-$) B cells. Naïve ($CD27^-$) B cells were cultured and stained with a fluorescein isothiocyanate-conjugated anti-IgG antibody on days 1, 4, 7, and 14. We compared cell-surface IgG levels of naïve ($CD27^-$) B cells treated with APRIL or not. These results indicate that APRIL exerted a significant effect on naïve ($CD27^-$) B-cell class switching compared with the controls on day 14 ($P < 0.01$). **(A)** Histograms showing expression of IgG determined on days 1, 4, 7, and 14 (median value; mean ± SED. BCR stimulation significantly altered naïve ($CD27^-$) B-cell class switching compared with controls on days 1, 4, 7, and 14 ($P < 0.01$, $P < 0.001$, $P < 0.001$ and $P < 0.0001$, respectively), and **(B)** fluorescence-activated cell sorting analysis revealed that BCR stimulation promoted the differentiation of naïve ($CD27^-$) B cells into PBs. **(C)** Comparison of IgG levels in culture supernatants of treated and untreated cells on days 7, 14, and 25. Naïve ($CD27^-$) B cells were collected from 10 healthy donors, and the data represent 10 independent experiments.

Figure 5. DNA synthesis by naïve ($CD27^-$) B-cell-derived PCs. **(A)** We cultured naïve ($CD27^-$) B cells for 10, 14, 21, 28, and 56 days and separated only $CD138^+$ PCs. All PCs were cultured in the presence of BrdU on the fifth day. These results represent experiments using cells pooled from five independent healthy donors. Ara-C (20 μg/ml) was added to cultures of naïve ($CD27^-$) B cells after 21 days. On days 28 and 56, cells were subjected to fluorescence-activated cell sorting analysis.We compared the phenotypes of cells treated or not with Ara-C. **(B)** Representative analyses of CD19, CD38, and CD138 expression of cells from the same donor on days 28 and 56. Viable PCs expressed $CD19^+CD38^+CD138^+$, PBs expressed $CD19^+CD38^{++}CD138^-$, and activated B cells expressed $CD19^+CD38^+CD138^-$. **(C)** The numbers of PBs and PCs were determined on day 28 in cultures treated or not with Ara-C on day 21 and the numbers of PCs was also determined on day 21 in cultures treated or not with Ara-C on day 14. In adittion, IgG levels **(D)** were determined on day 28 in cultures treated or not with AraC on day 21. **(E)** We estimated antibody (Ab) production per naïve ($CD27^-$) B-cell-derived PC per day. Naïve ($CD27^-$) B cells were cultured for 28 and 56 days, and we replenished the medium and cultured them for 1 day to calculate the IgG production per day. We calculated this value by dividing the amount of IgG in the culture supernatant by the number of viable PCs at the time of collection of the culture supernatant. Cultures were treated or not with Ara-C, and we compared Ab production per naïve ($CD27^-$) B-cell-derived PC per day on days 28 and 56 between the Ara-C-treated(*) and untreated cultures. Ara-C did not affect IgG levels on days 28 and 56 compared with controls.

Naïve ($CD27^-$) B cells were collected from 10 healthy donors, and data are representative of 10 independent experiments.

Figure 6. Expression of genes encoding transcription factors involved in the differentiation of naïve ($CD27^-$) B cells into PCs. Naïve ($CD27^-$) B cells were cultured with APRIL for 0, 10, 25, and 35 days, and then the levels of *BCL6*, *PAX5*, *XBP1*, and *PRDM1* mRNAs were evaluated

using quantitative real-time polymerase chain reaction **(A, B, C, D)**. Naïve ($CD27^-$) B cells were collected from 10 independent healthy donors, and the data are representative of 10 independent experiments. The graphs show the mean ± SEM. Significance was tested using a paired *t* test. Significant changes from baseline are indicated with asterisks (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$).

Figure 7. Effects of antibodies against APRIL receptors on the phenotype of naïve ($CD27^-$) B cells. **(A)** We performed fluorescence-activated cell sorting (FACS) analyses to estimate the levels of cell-surface B-cell mature antigen (BCMA) and transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) expressed by naïve ($CD27^-$) B cells on days 7, 14, and 21. BCMA expression increased up to day 14 and was changed little on day 21. TACI levels increased up to day 14 and then decreased to their initial levels. **(B)** We added an anti-APRIL mAb and an anti-APRIL receptor mAb or mouse IgG1κ on days 0 and 7 and then determined the number of naïve ($CD27^-$) B-cell-derived PCs on day 25. **(C)** Number of viable PCs on day 25 after treatment on day 14. **(D)** On day 25, we estimated IgG and IgM levels in cultures treated or not with an anti-APRIL receptor mAb on day 14. **(E)** FACS analysis shows that the anti-BCMA antibody altered cell differentiation on day 25. Naïve ($CD27^-$) B cells were collected from 10 healthy donors, and the data represent 10 independent experiments. The graphs show the mean ± SED. Significance was tested using a paired *t* test. Significant changes from baseline are indicated by an asterisk (*$P < 0.05$).

Figure
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Fig 1

Day 0
Naïve B-cells

Day 0
Memory B-cells

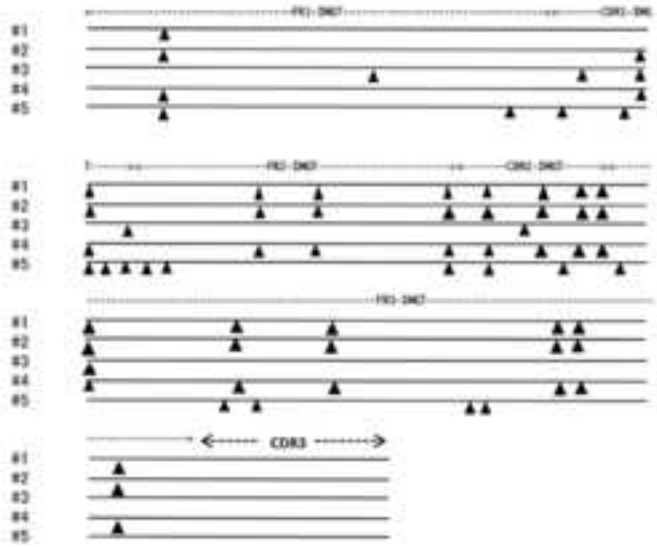

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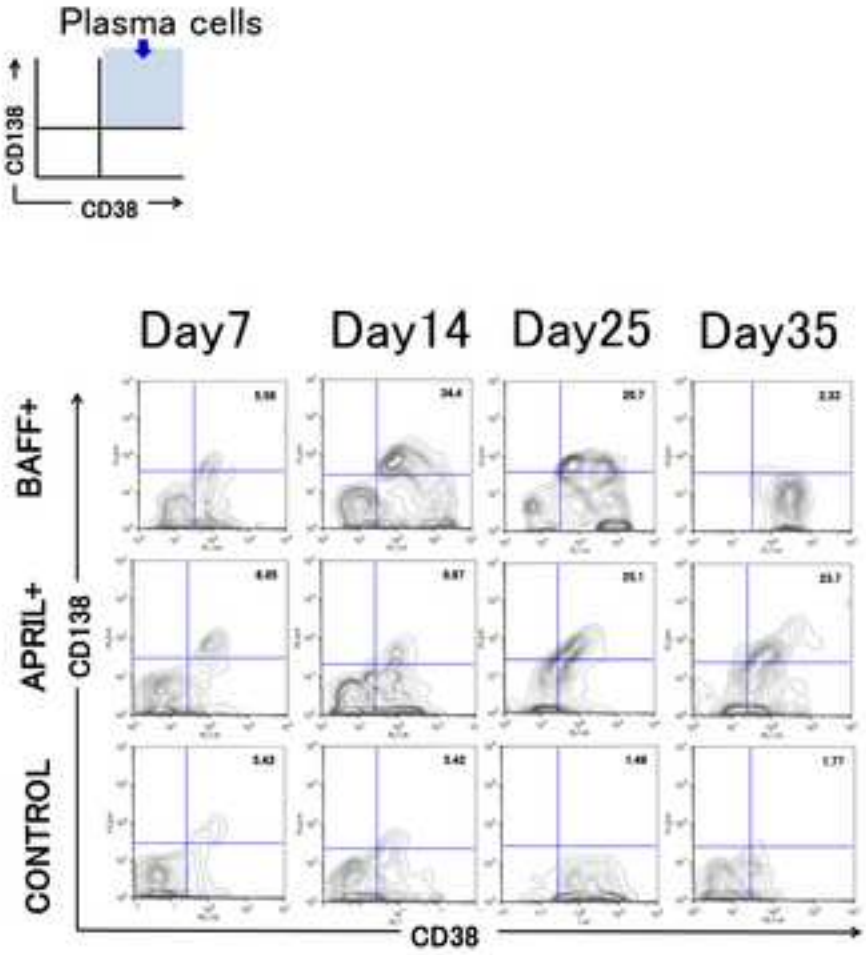
Fig 2

A



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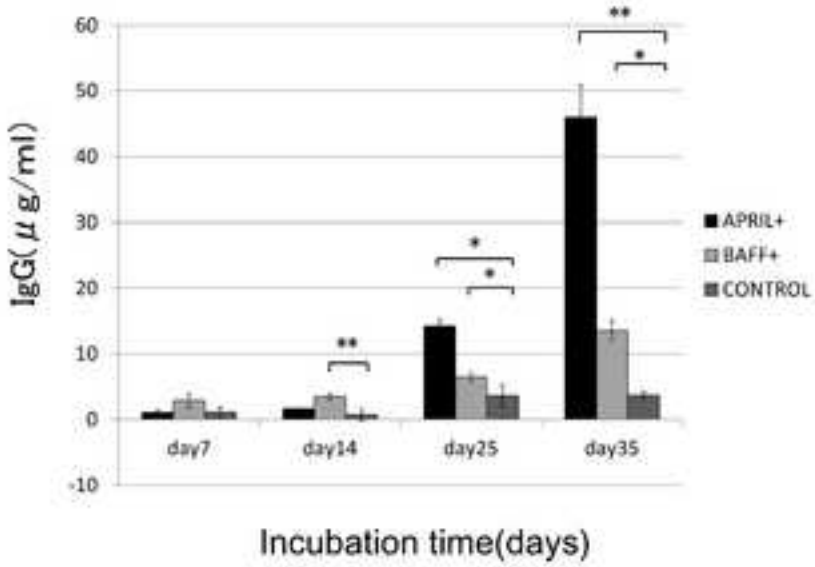

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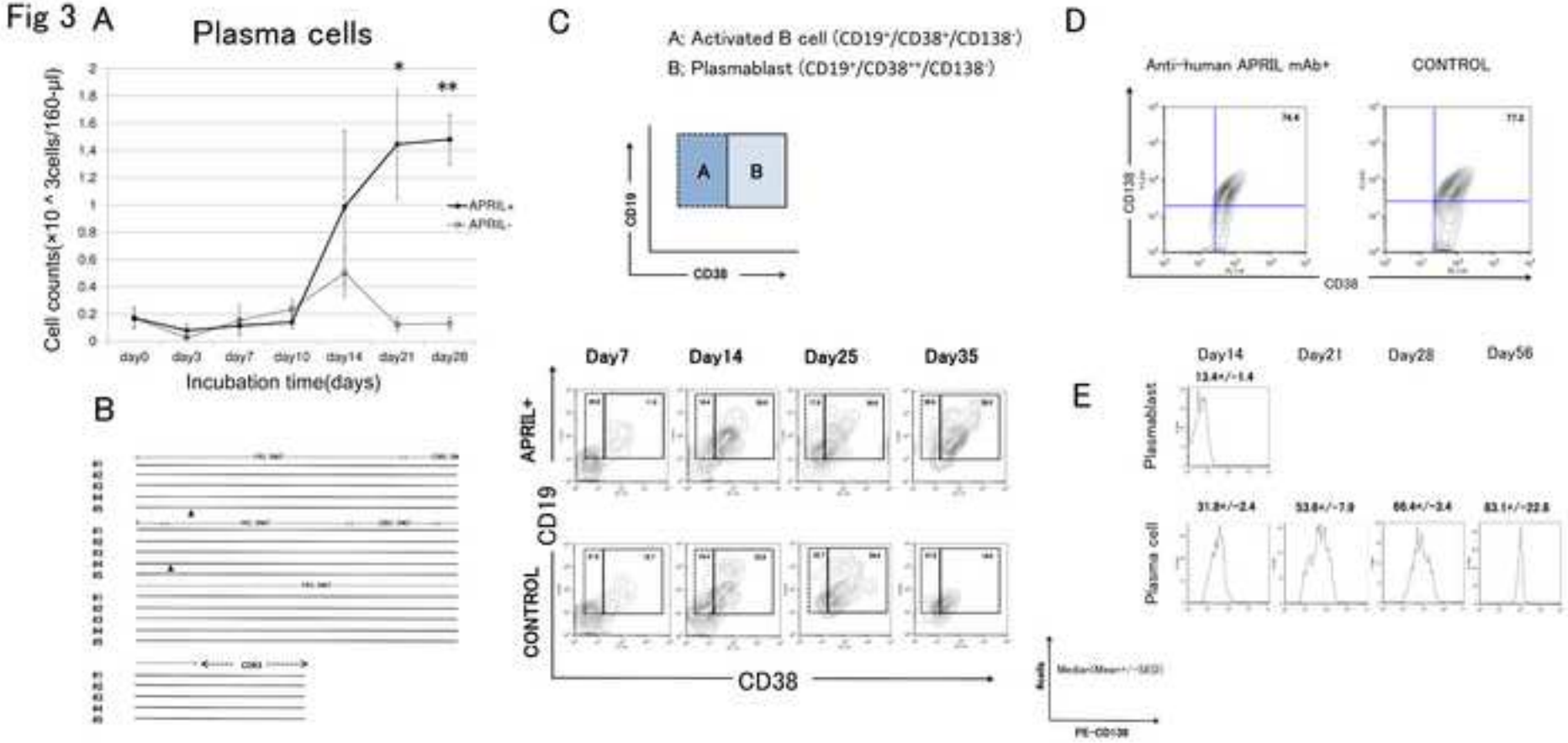

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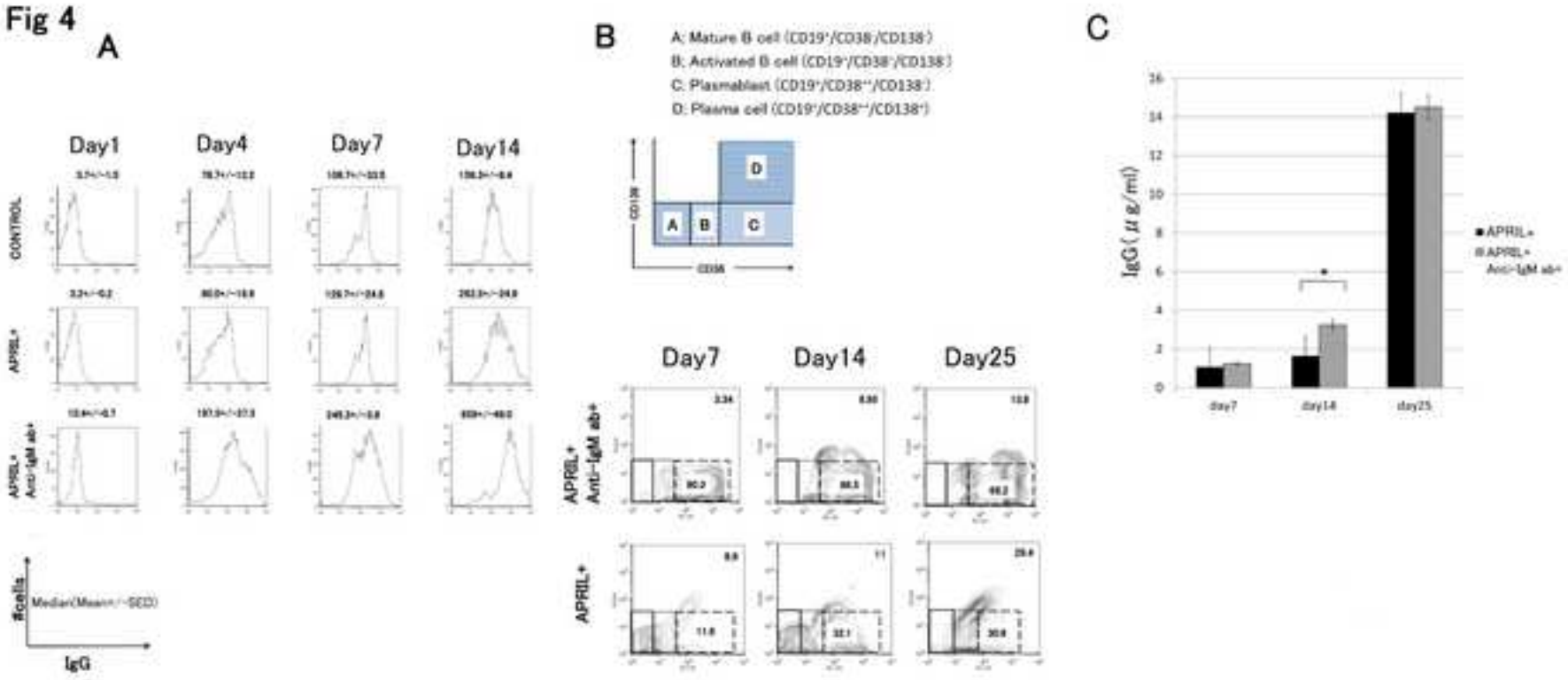

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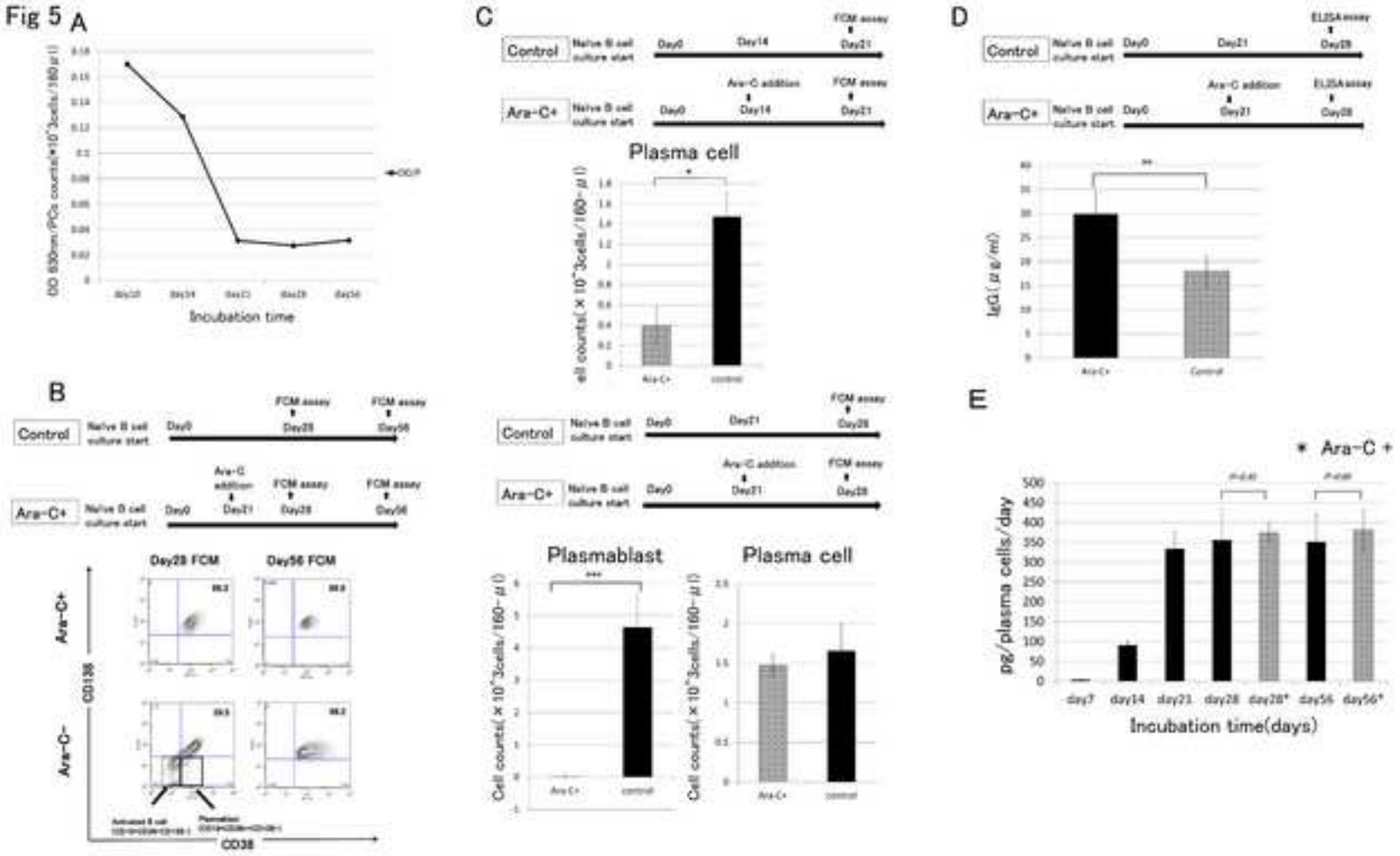

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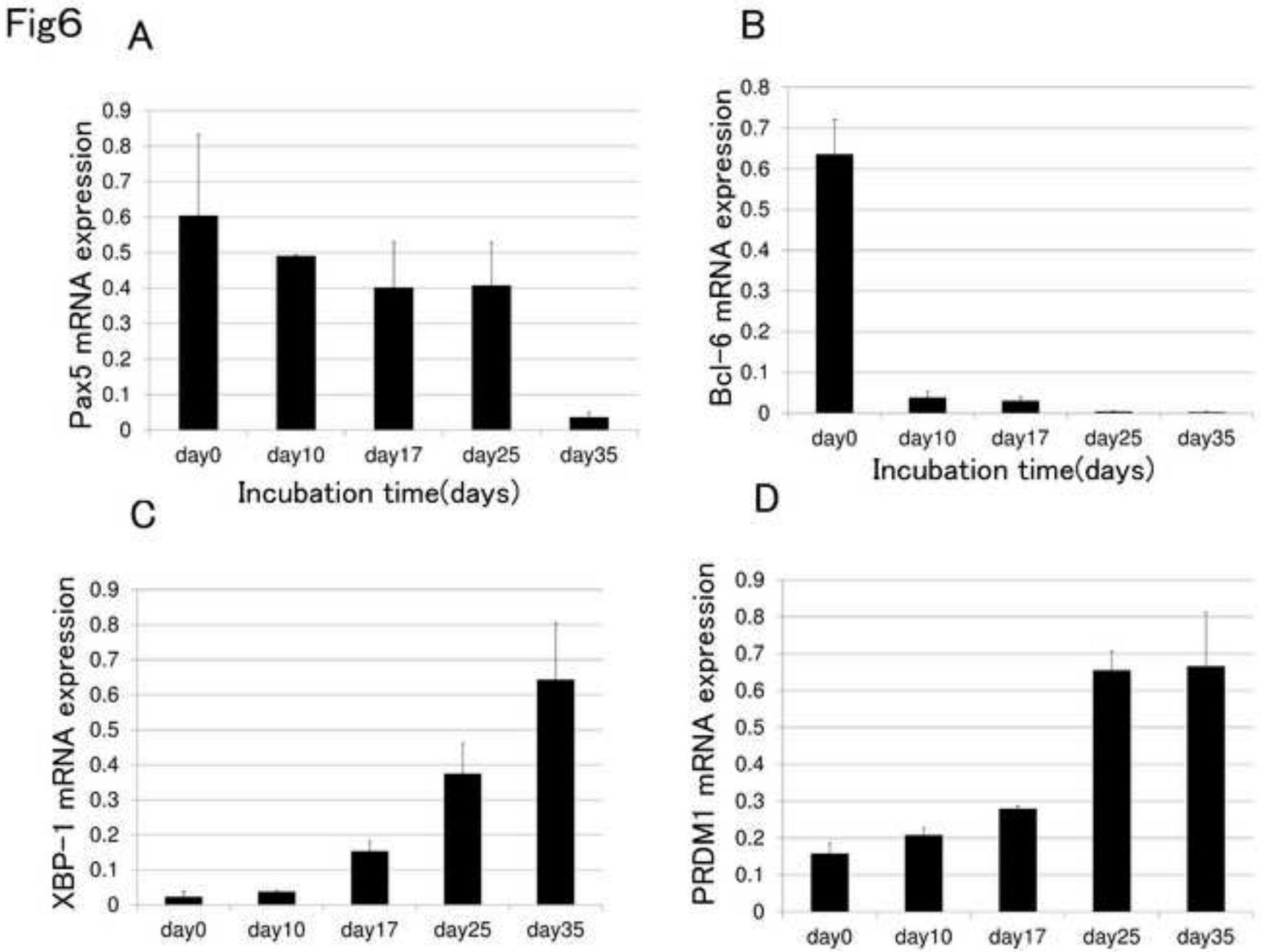

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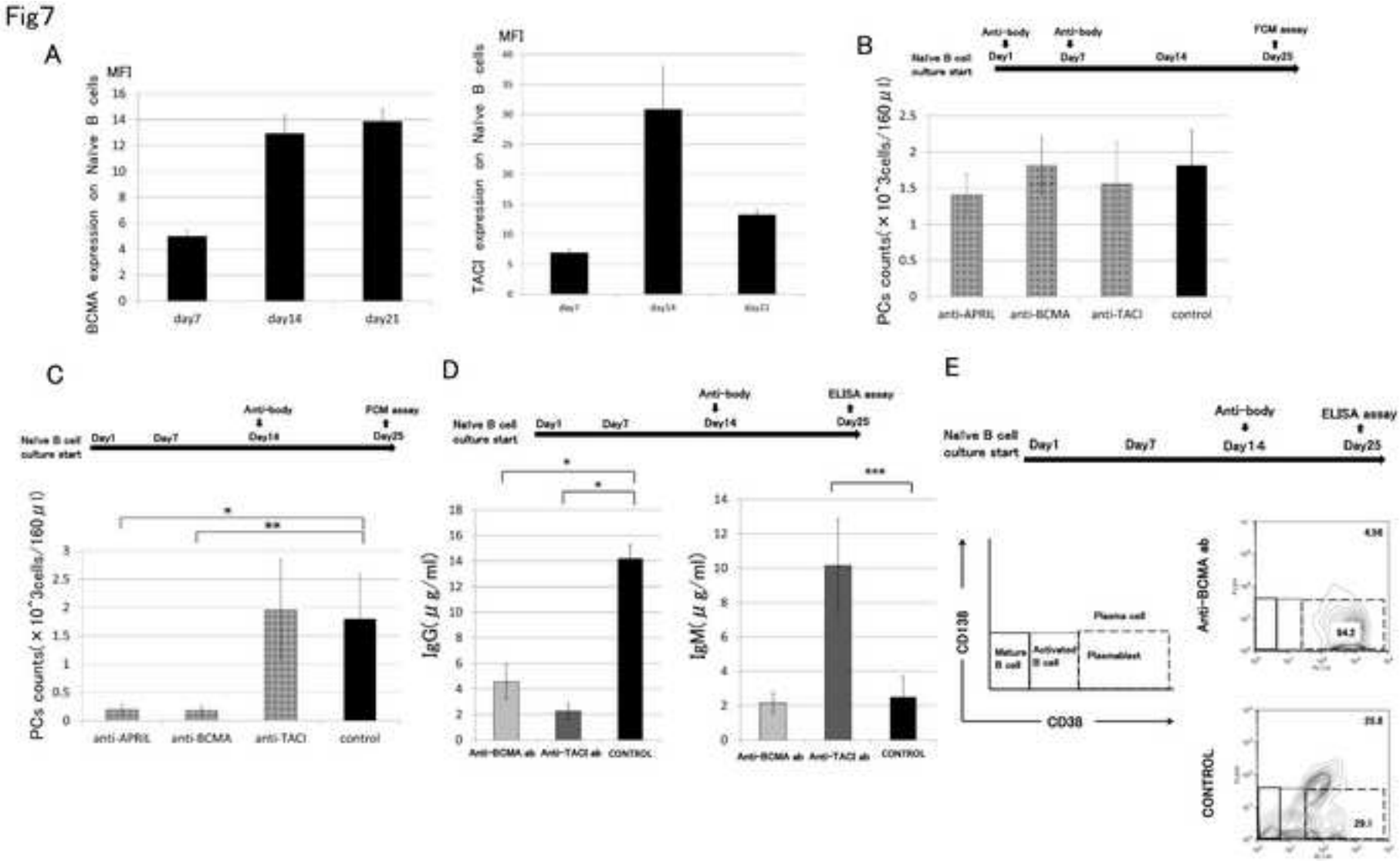

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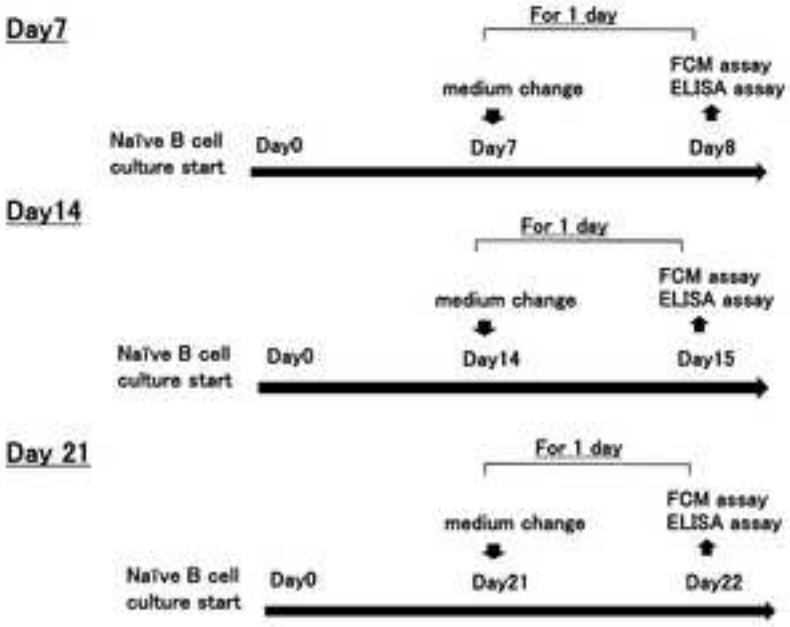

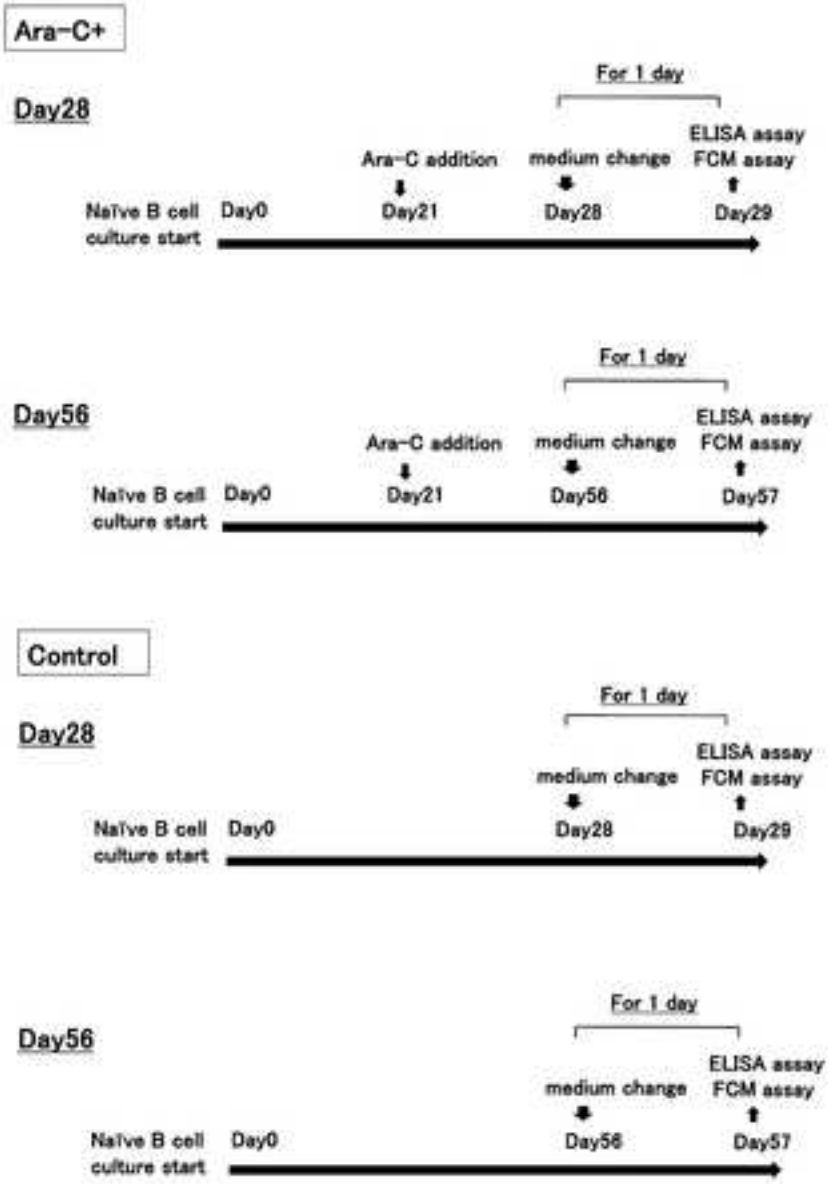

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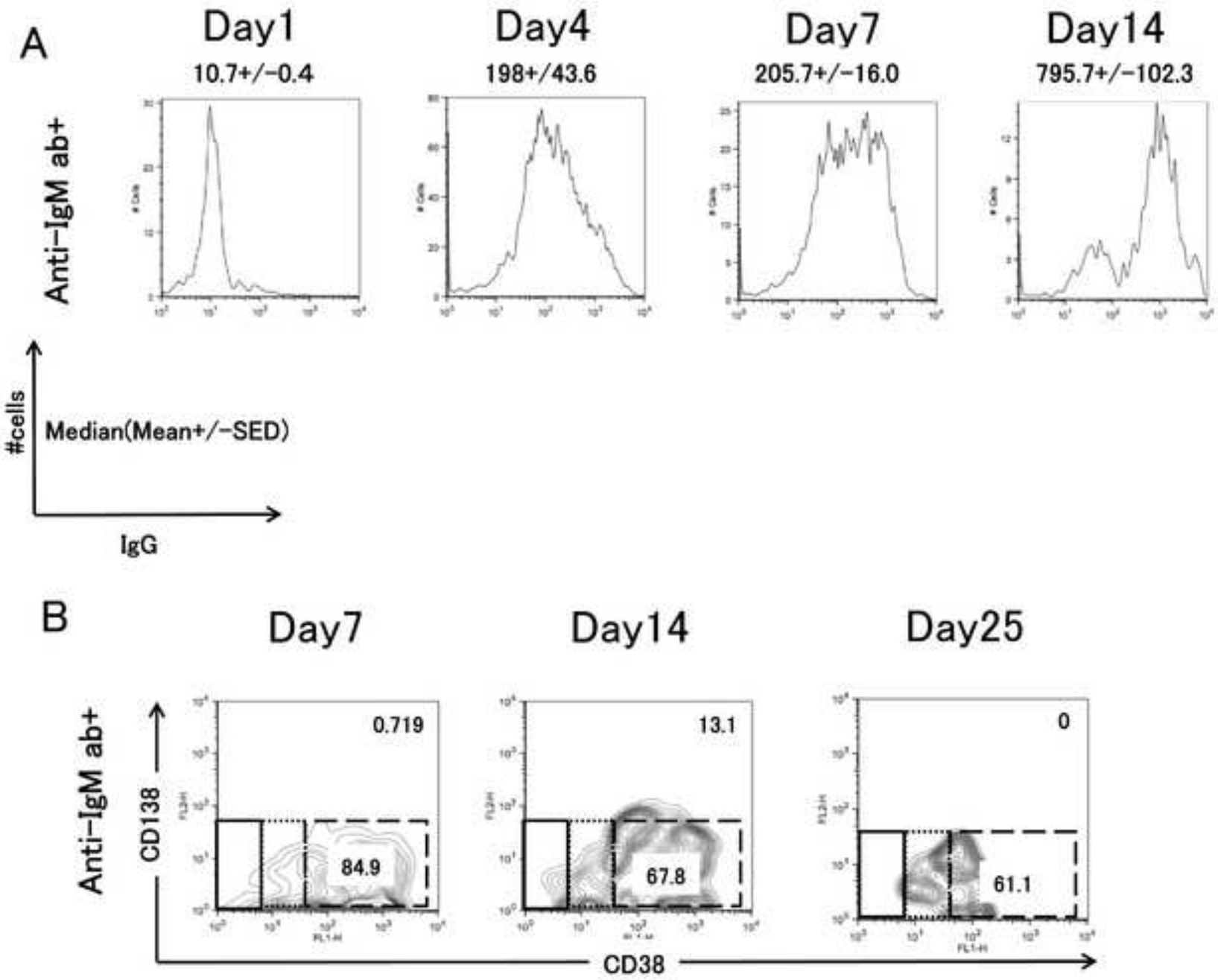
Supplementary figure