

Original Article



Efficient *In Vitro* Plasma Cell Differentiation by B Cell Receptor Activation and Cytokine Stimulation

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Conflict of Interest

The authors have no financial conflicts of interest.

Abbreviations

ASC, Ab-secreting cell; BCR, B cell receptor; GC, germinal center; LARC, Laboratory Animal Resource Center; LCMV, lymphocytic choriomeningitis virus; NP, nitrophenol; PBS-T, PBS containing 0.05% Tween-20; PC, plasma cell; RT, room temperature.

ABSTRACT

Plasma cells (PCs) constitute a small proportion of B cells, limiting their biochemical characterization. An *in vitro* culture system that reliably generates PCs could provide an alternative method to obtain PCs for further analysis and manipulation. To date, most *in vitro* PC differentiation methods rely on B cell receptor (BCR)-independent stimulants, including TLR ligands, CD40L, and cytokines. However, these methods do not fully recapitulate the natural T cell-dependent PC differentiation process, in which BCR activation is the initial events. In this study, we established an efficient *in vitro* PC differentiation method incorporating BCR stimulation. Naïve B cells were first stimulated with anti-IgM and anti-CD40 Abs, followed by stimulation with various cytokines. By screening cytokines known to participate in PC differentiation *in vivo*, we identified that the combination of IL-4 and IL-5 induced the most efficient PC differentiation. The *in vitro* generated PCs highly expressed PC-associated surface markers and regulatory genes. Additionally, they secreted high amounts of IgM and IgG Abs. Moreover, retroviral transduction of B cells resulted in efficient target gene expression in PCs. Our new method closely mimics natural PC differentiation and effectively generates a large quantity of PCs for various applications, including elucidating the molecular mechanisms underlying PC differentiation.

Keywords: B-lymphocytes; Plasma cell; B cell receptor; *In vitro* differentiation

INTRODUCTION

B cells confer host protection against intruding pathogens by producing neutralizing Abs. For T cell-dependent Ab production, naïve B cells sense specific Ags from invading pathogens via B cell receptors (BCRs). They then internalize the BCR-Ag complexes and present the Ags via MHC class II to cognate CD4 T cells, which “help” the B cells through CD40L expression and cytokine secretion (1-3). The combination of BCR-mediated signaling, CD40L-induced CD40 activation, and cytokine receptor signaling induces clonal expansion, class switch recombination, and differentiation of B cells into Ab-secreting cells (ASCs). In the early phase of infection, differentiation into ASCs primarily occurs outside B cell follicles, leading to the extrafollicular generation of short-lived plasmablasts or plasma cells (PCs). In contrast, activated B cells and T cells that enter B cell follicles form germinal centers

Author Contributions

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(GCs), where B cells undergo cycles of proliferation, somatic hypermutation, and selection, eventually differentiating into affinity matured, long-lived PCs or memory B cells. The former migrates into the bone marrow and provides long-term protection against reinfection by the same pathogens through continuous Ag-specific Ab production (1-4). As improper PC differentiation can result in detrimental outcomes, such as the failure to overcome infections, development of multiple myeloma, and Ab-mediated autoimmune diseases, it is critical to thoroughly understand the molecular mechanisms governing PC differentiation.

For decades, research has focused on identifying the key modulators and molecular mechanisms that drive PC differentiation (2,5). However, our understanding of the PC differentiation process remains incomplete. Unlike other B cell subsets, PCs exist at very low frequencies in the bone marrow and peripheral organs, making it difficult to study them using biochemical or cell biological approaches. Moreover, a single pathogen infection event leaves only a small number of pathogen-specific PCs in the bone marrow (6-8). For example, a single lymphocytic choriomeningitis virus (LCMV) infection induces only 2×10^4 to 3×10^4 LCMV-specific bone marrow-residing ASCs in mice (8), making it even more challenging to study an Ag-specific PC population within the total PC pool. Additionally, previous studies have identified various PC populations with distinct surface phenotypes and functions, depending on numerous factors such as B cell origin, Ag type, and the cytokine milieu (9). Recent advances in single-cell RNA sequencing technologies have revealed an even more diverse landscape of heterogeneous PC populations (10-12), further highlighting the difficulties in obtaining homogenous PC populations for characterization.

One approach to overcoming these limitations is the use of BCR transgenic mice, where every B cell expresses the same BCRs specific to targeted Ags such as ovalbumin, nitrophenol (NP), hen egg lysozyme, and chicken gamma globulin (13). Reporter mice expressing a fluorescent reporter gene under the promoters of genes of interest, such as *Aicda* (encoding AID) and *Prdm1* (encoding BLIMP-1), also allow for tracking and investigating PC differentiation *in vivo* (14,15). However, experiments using these genetic models are time-consuming and costly. The *in vitro* B cell culture system offers an attractive alternative, as it enables the production of large numbers of PCs required for subsequent assays in a relatively short time period. Additionally, *in vitro* systems allow researchers to easily manipulate culture conditions and monitor B cell populations over time, facilitating the identification of critical exogenous factors and close examination of the time-dependent PC differentiation process. Moreover, genetic modification of B cells during *in vitro* culture can enable the identification of endogenous factors required for PC differentiation and function.

Several widely used methods for *in vitro* PC differentiation rely on the reactivation of memory B cells or GC B cells isolated from human peripheral blood mononuclear cells or tonsils (16-19). Moreover, most B cell culture methods for *in vitro* PC differentiation use BCR-independent stimulants, including cytokines, CD40L, BAFF, and Toll-like receptor ligands such as LPS and CpG (20-23). These methods do not effectively recapitulate the *in vivo* PC differentiation process, where BCR-mediated activation of naïve B cell is the first step.

In this study, we established a simple but effective *in vitro* PC differentiation method incorporating BCR activation. Our method involves 2-step culture process. First, naïve follicular B cells are activated and induced to proliferate through stimulation with anti-IgM F(ab')₂ and anti-CD40 Abs. Next, the activated B cells undergo class switch recombination and terminal PC differentiation via cytokine stimulation. This method effectively generates

PCs that express canonical PC marker genes and secrete isotype-switched immunoglobulins. Additionally, we demonstrated that retroviral gene transduction during *in vitro* culture enables highly efficient genetic manipulation of PCs. This new *in vitro* PC differentiation method is expected to facilitate the biochemical analysis of PCs and the elucidation of the molecular mechanisms underlying PC differentiation.

MATERIALS AND METHODS

Mice and cell lines

Wild type C57BL/6 mice were purchased from DBL (Eumseong, Korea) or provided by the KAIST Laboratory Animal Resource Center (LARC), and housed in the specific pathogen free facility of KAIST LARC. All mouse experiments were approved by the Institutional Animal Care and Use Committee of KAIST. The CD40LB feeder cell line (23) was kindly provided by Seung Goo Kang (Kangwon National University, Korea) and maintained in DMEM (Welgene, Gyeongsan, Korea) supplemented with 10% fetal bovine serum (FBS, Welgene) and 1% penicillin/streptomycin (Welgene).

Preparation of mouse naïve B cells

The spleens of wild type C57BL/6 mice were mechanically disrupted using a mesh strainer and syringe plunger. Splenocytes were incubated in ACK lysis buffer for 3 min at room temperature (RT) to remove red blood cells. Naïve follicular B cells were purified by negative selection using BD IMag Cell Separation Magnets (BD Bioscience, USA) following the manufacturer's instructions. Briefly, splenocytes were sequentially labeled with a mixture of biotin-conjugated Abs (CD4, CD5, CD8, CD9, CD11b, CD11c, CD43, CD93, TCR β , TER119) and BD IMag Streptavidin Particles Plus-DM (BD Biosciences, Franklin Lakes, NJ, USA), and applied to the magnet separation. Cells that were not retained by the magnet were collected and resuspended in RPMI 1640 medium (Welgene) supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT, USA), 10 mM HEPES (Welgene), 2 mM L-glutamine (Welgene), 1 mM sodium pyruvate (Welgene), 55 μ M β -mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), and 1% penicillin/streptomycin (Welgene). Cell purity of naïve B cells was assessed by flow cytometry and confirmed to be >95%.

In vitro PC differentiation

Naïve B cells were plated in 96-well cell culture plate at a density of 1×10^6 cells/ml and stimulated with 10 μ g/ml goat anti-mouse IgM F(ab')₂ (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and 1 μ g/ml anti-CD40 Ab (HM40-3; eBioscience, San Diego, CA, USA) for 3 days. Cells were then washed twice with PBS (Welgene) and reseeded in 96-well culture plate at a density of 1×10^6 cells/ml. Cells were cultured with cytokines (IL-2: 100 ng/ml, IL-4: 10 ng/ml, IL-5: 2 ng/ml, IL-10, 50 ng/ml, IL-21: 50 ng/ml; all from R&D Systems, Minneapolis, MN, USA) for additional 4 days.

PC differentiation using the CD40LB feeder cell line was performed according to the method reported by Nojima et al. (23). Briefly, CD40LB feeder cells were plated in 6-cm tissue culture dish and γ -irradiated with 100 Gy. Naïve follicular B cells were then added to the plates at a density of 2×10^4 cells/ml and stimulated with 1 ng/ml of IL-4 (R&D Systems) for 4 days. B cells were then harvested, reseeded in new culture dish in the presence of irradiated CD40LB feeder cells at a density of 2×10^4 cells/ml, and stimulated with 10 ng/ml of IL-21 (R&D Systems) for additional 4 days.

Flow cytometry

Cells were first stained with Ghost Dye (Tonbo Bioscience, San Diego, CA, USA) and then with following fluorochrome-conjugated Abs: B220, CD138, CD44, CD93, TACI, IgD, and MHCII. After washing with FACS buffer (PBS supplemented with 2% BSA), cells were analyzed using an LSR Fortessa X-20 (BD Biosciences) or FACSymphony A5 (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Intracellular immunoglobulin assay

Cells were first stained with Ghost Dye (Tonbo Bioscience) and then with fluorochrome-conjugated B220, and CD138 Abs. After washing with FACS buffer, cells were fixed and permeabilized with 3.7% formaldehyde and 0.5% saponin in FACS buffer, respectively. After staining intracellular immunoglobulins with fluorochrome-conjugated Abs (IgG1, IgM, IgA), cells were analyzed using an LSR Fortessa X-20 or FACSymphony A5. Data were analyzed using FlowJo software (Tree Star).

Immunoglobulin ELISA

Ninety-six-well immunoplates (SPL, Pocheon, Korea) were coated with anti-mouse Ig (SouthernBiotech, Birmingham, AL, USA) for overnight at 4°C, washed twice with PBS containing 0.05% Tween-20 (PBS-T), and blocked with PBS-T supplemented with 2% BSA for 1 h at RT. After washing twice with PBS-T, the plates were incubated with culture supernatant for 1 h at RT, washed 5 times with PBS-T, and incubated with anti-Ig-HRP (SouthernBiotech) for 1 h at RT. After washing 5 times with PBS-T, tetramethylbenzidine solution (SurModics, Eden Prairie, MN, USA) was added as substrate, and the HRP enzyme reaction was stopped by adding 0.5 M H₂SO₄. Absorbance at 450 nm was read using a SpectraMax M2 Microplate Reader (Molecular Devices, San Jose, CA, USA).

RNA preparation and qPCR assay

Cell lysis and RNA preparation was performed using QIAzol lysis reagent (QIAGEN, Hilden, Germany) following the manufacturer's instruction. RNA samples were then subjected to cDNA synthesis using GoScript Reverse Transcriptase, ribonuclease inhibitor, dNTP, MgCl₂, and oligo(dT)15 primer (all from Promega, Madison, WI, USA) following the manufacturer's instructions. Using cDNA as templates, qPCR analysis of genes of interest was performed with SYBR Green Realtime PCR Master Mix (TOYOBO, Tokyo, Japan) and a CFX Connect Real-Time System (Bio-Rad, Hercules, CA, USA). Gene expression levels were calculated based on the comparative C_q method and normalized to *Ubc* gene expression. The following primers are used for qPCR analysis.

Pax5: GTATTATGAGACAGGAAGC, CTGTTGATGGAGCTGACG;
Bach2: GTCGGAGGAAGCTGGACTG, GAGGCAGGAGTTGTCCAGG;
Bcl6: CCGGCACGCTAGTGATGTT, TGTCTTATGGGCTCT-CTGCT;
Irf4: TCTTCAAGGCTTGGGCATTG, CACATCGTAATCTTGTCTTCCAAGTAG;
Aicda: GCCACCTTCGCAACAAGTCT, CCGGGCACAGTCATAGCAC;
Prdm1: TTGAGATTGCTGTGCTGCT, TCTCCAACCTGAAGGTCCAC;
Xbp1: TGGACTCTGACACTGTTGCCTC, TAGACCTCTGGGAGTTCCTCCA;
Jchain: TGACGACGAAGCGACCATTC, TTC-GGGACAACAATTCGGA;
Ubc: CCCAGTGTTACCACCAAGAAG, CCCCATCACACCCAAGAACA.

Retroviral transduction

HEK293T cells were transfected with the retroviral expression vector, pMSCVpuro-eGFP, along with pCL-Eco vector (24) for retrovirus production. Twelve hours after the transfection,

the media was changed to fresh media, and virus-containing culture supernatant was collected 48 h later and spun at 3,000 rpm for 5 min to clear debris. For retroviral infection, the culture supernatant was removed and saved from B cells 24 h post α -IgM/CD40 (10 μ g/ml anti-mouse IgM and 1 μ g/mL anti-CD40 Ab) stimulation. The B cells were then treated with the virus solution pre-mixed with 8 μ g/ml polybrene (Sigma-Aldrich) and spun at 2,200 rpm for 90 min. Immediately after the spin infection, the virus-containing supernatant was exchanged with the saved culture supernatant containing α -IgM/CD40. After 2 days of culture, the cells were stimulated with IL-4 and IL-5 for additional 4 days as described in the in vitro PC differentiation method above.

Statistical analysis

Statistical analyses were performed using an unpaired Student's *t*-test to evaluate significant differences between experimental groups using GraphPad Prism software (GraphPad, San Diego, CA, USA).

RESULTS

Efficient differentiation of BCR-activated B cells to PCs by combined stimulation of IL-4 and IL-5

To mimic Ag-induced BCR activation and CD40L-induced CD40 activation, we first stimulated naïve follicular B cells, which were purified from mouse spleens and seeded at a density of 1×10^6 cells/ml in 96-well plates (2×10^5 cells/well), using a combination of anti-IgM F(ab')₂ and anti-CD40 Abs (α -IgM/CD40) (**Fig. 1A**). As previously reported (25), α -IgM/CD40 stimulation resulted in robust proliferation of naïve B cells, whereas α -CD40 alone was insufficient to induce B cell proliferation (**Fig. 1B**). The total number of B cells increased approximately 1.6-fold following α -IgM/CD40 stimulation for 3 days, while the cell number did not significantly change in the group treated with anti-CD40 alone.

After washing out the α -IgM/CD40, the activated B cells were reseeded at a density of 1×10^6 cells/ml and then treated with various cytokines for 4 days. We selected IL-2, IL-4, IL-5, IL-10, and IL-21 based on previous reports on the PC differentiation of human or mouse B cells (26-29). Each cytokine was tested individually or in different combinations, and the proportion of B220⁺CD138⁺ PCs among live cells was analyzed by flow cytometry (**Fig. 1A**).

IL-4 or IL-5 alone did not efficiently induce the differentiation of activated B cells into B220⁺CD138⁺ cells (**Fig. 1C**). However, the combination of IL-4 and IL-5 (IL-4/5) resulted in high proportions of B220⁺CD138⁺ PCs in both α -IgM/CD40- and α -CD40-activated groups (**Fig. 1C**). The efficiency of IL-4/5-mediated PC differentiation was higher in α -IgM/CD40-activated cells compared to α -CD40-activated cells (**Fig. 1C**). Additionally, IL-4/5 stimulation led to cell proliferation in the α -IgM/CD40-activated group, whereas the cell number did not increase in the α -CD40-activated group (**Fig. 1B**).

The addition of IL-21 to the combination of IL-4/5 (IL-4/5/21) further increased the proportions of B220⁺CD138⁺ PCs in both α -IgM/CD40- and α -CD40-activated groups (**Fig. 1C**), but it also caused cell death in both groups (**Fig. 1B**). As a result, the final yield of B220⁺CD138⁺ PCs was the highest under the condition where B cells were activated with α -IgM/CD40, followed by IL-4/5-stimulation (**Fig. 1D**). This condition produced approximately 3.5×10^5 B220⁺CD138⁺ PCs on day 7, starting from 2×10^5 naïve B cells on day 0. The efficiency of IL-4/5- and IL-

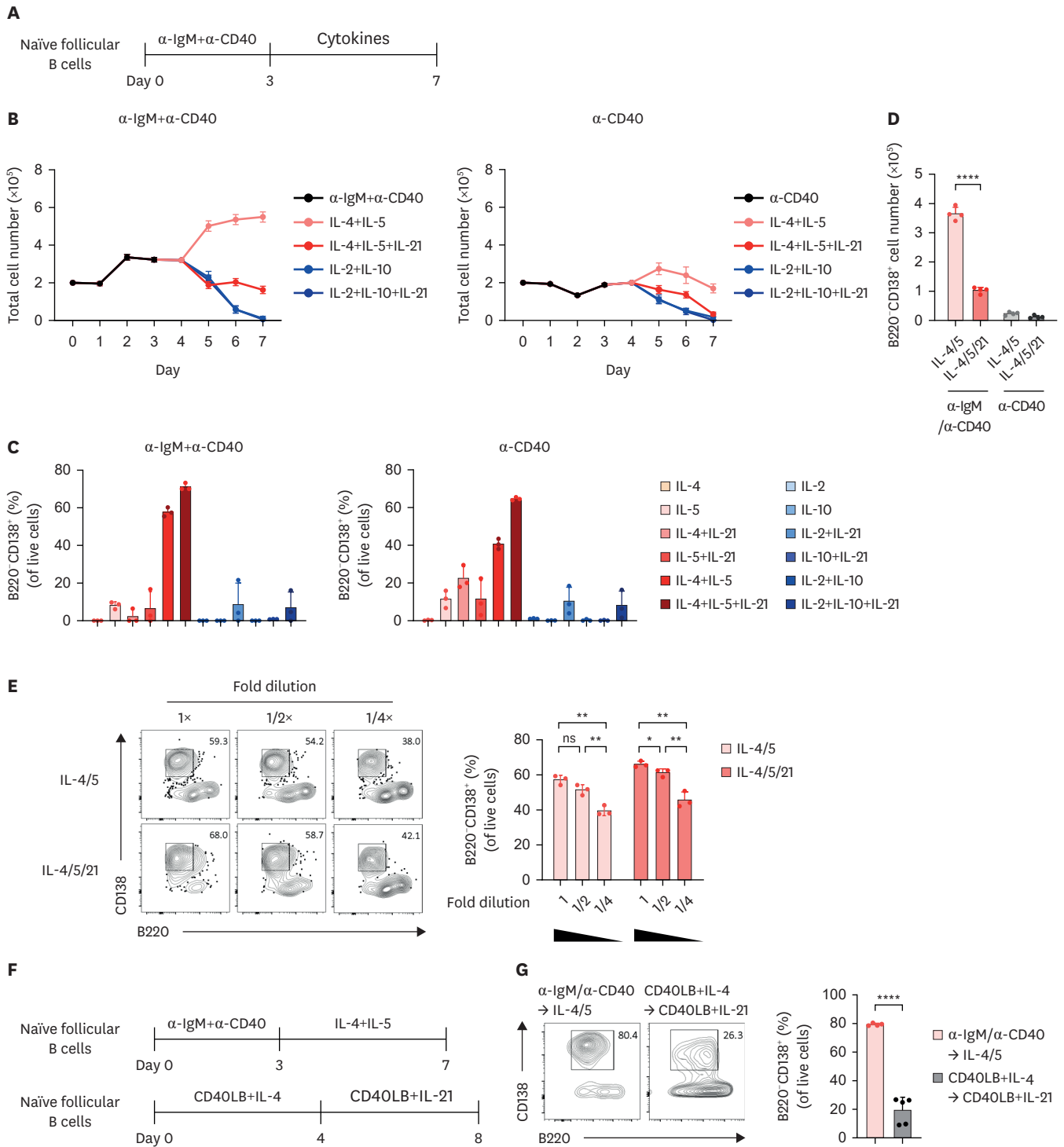


Figure 1. Activation of naïve follicular B cells using α -IgM/ α -CD40, followed by IL-4/5 or IL-4/5/21 stimulation, induces efficient *in vitro* plasma cell differentiation of murine naïve follicular B cells. (A) Experimental scheme of the *in vitro* PC differentiation assay. Naïve follicular B cells were activated with either α -IgM/ α -CD40 or α -CD40 alone for 3 days, followed by stimulation with the indicated cytokines for additional 4 days. (B) Total cell numbers on each day of *in vitro* PC differentiation. (C) Proportions of B220⁺CD138⁺ cells on day 7. (D) B220⁺CD138⁺ B cell numbers on day 7. (E) Proportions of B220⁺CD138⁺ cells after PC differentiation with different cytokine concentrations. (F) Experimental schemes of *in vitro* PC differentiation using α -IgM/ α -CD40 + IL-4/5 and CD40LB + IL-4 + IL-21. (G) Proportions of B220⁺CD138⁺ cells on the final day of culture. Data are presented as mean \pm SD. Unpaired, 2-tailed Student's *t*-test (**p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001).

4/5/21-induced PC differentiation of α -IgM/CD40-activated cells was dependent on cytokine concentration and gradually decreased with the dilution of cytokines (**Fig. 1E**). In contrast to IL-4 and IL-5, IL-2 and IL-10, either alone or in combination with IL-21, barely induced PC differentiation (**Fig. 1C**). Moreover, the combination of IL-2 and IL-10, as well as the combination of IL-2, IL-10, and IL-21, induced rapid cell death in both α -IgM/CD40- and α -CD40-activated B cells (**Fig. 1B**).

The CD40LB feeder cell line, which overexpresses CD40L and BAFF, is frequently used for *in vitro* differentiation of GC B cells and PCs in combination with IL-4 and IL-21 (23). Therefore, we compared the PC differentiation efficiencies between our newly established method and the published method using the CD40LB cell line (23). For PC differentiation using the CD40LB cell line, naïve follicular B cells were cultured together with the CD40LB cell line for 8 days. IL-4 and IL-21 were added to the culture condition during the first and the last 4 days, respectively (**Fig. 1F**). Although B220⁻CD138⁺ PCs were clearly observed in the culture condition using the CD40LB cell line, our PC differentiation method using α -IgM/CD40 and IL-4/5 yielded much higher frequencies of PCs (**Fig. 1G**).

Expression of surface markers on *in vitro* differentiated PCs

Next, we examined the expression of surface markers in B cells cultured under our PC differentiation conditions for 7 days. Compared to B220⁻CD138⁻ B cells (undifferentiated B cells), B220⁻CD138⁺ PCs expressed significantly elevated levels of surface markers typically associated with PCs, including CD44, CD93, and TACI, in both IL-4/5 and IL-4/5/21 stimulation conditions (**Fig. 2A**). In contrast, expression of IgD and MHCII was highly downregulated in the B220⁻CD138⁻ population under both conditions. These results suggest that B220⁻CD138⁺ cells generated using our *in vitro* culture conditions closely mimic the surface expression profile of PCs found *in vivo*.

In line with the higher proportion of B220⁻CD138⁺ PCs in the IL-4/5/21 stimulation condition compared to the IL-4/5 condition (**Fig. 1C**), the proportions of B220⁻, CD138⁺, CD44⁺, CD93⁺, TACI⁺, IgD⁻, and MHC II⁻ cells were all higher in the IL-4/5/21 stimulation group (**Fig. 2B**). Nonetheless, expression levels of surface markers were comparable in B220⁻CD138⁺ cells from IL-4/5 and IL-4/5/21-treated groups, except for CD93, whose expression was slightly higher in IL-4/5/21-stimulated cells (**Fig. 2C**). These results suggest that the characteristics of PCs generated by IL-4/5 and IL-4/5/21 stimulation are similar.

We also investigated the dynamics of PC differentiation and surface marker expression over time. The B220⁻CD138⁺ population was absent during the first 3 days of α -IgM/CD40 activation but began to emerge after cytokine stimulation on day 5 and significantly increased by day 7 under both IL-4/5 and IL-4/5/21 stimulation conditions (**Fig. 3A**). Culturing cells with cytokines beyond day 7 did not increase the proportion of B220⁻CD138⁺ population (data not shown). Correspondingly, the decrease in B220 expression levels and the increase in CD138 levels were first observed on day 5 and were significantly enhanced by day 7 (**Fig. 3B and C**). On the other hand, increased expression of CD44, CD93 and TACI, as well as decreased expression of IgD, were noticeable starting from day 3, while the decrease in MHC II was only detected on day 7. Generally, these changes in surface marker expression were more pronounced in IL-4/5/21-stimulated cells compared to IL-4/5-stimulated cells. Notably, the expression of IgD and MHC II was transiently increased in IL-4/5-stimulated cells on day 5 (**Fig. 3B and C**). The reason for this is not clear, but it appears to be associated with the transient cell proliferation induced by IL-4/5 stimulation (**Fig. 1B**).

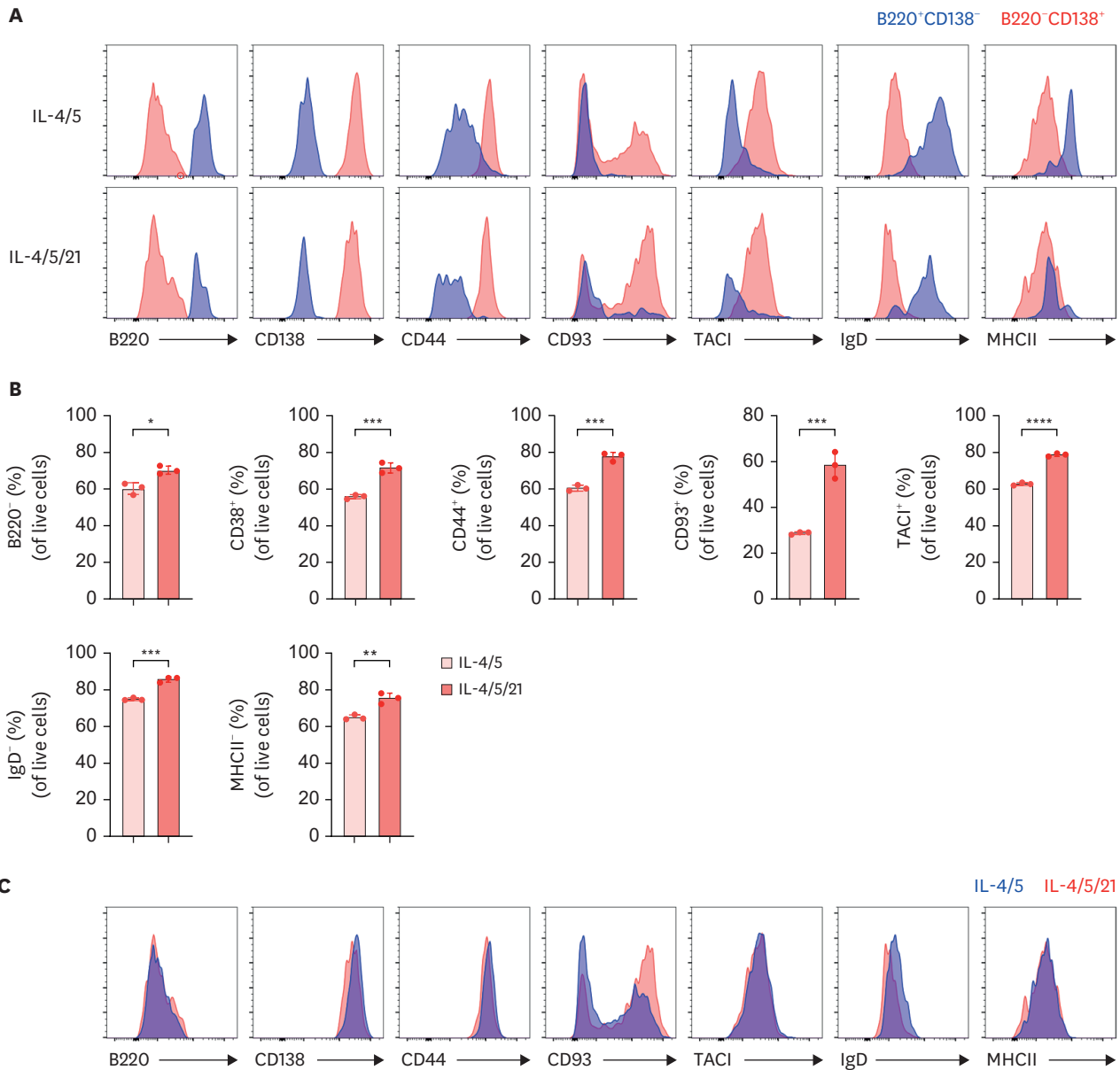


Figure 2. *In vitro* PC differentiation by α -IgM/ α -CD40 and cytokine stimulation induces the expression of plasma cell-associated surface markers. (A) Expression levels of B220, CD138, CD44, CD93, TAC1, IgD, and MHCII in B220⁺CD138⁻ and B220⁻CD138⁺ cells on day 7 of *in vitro* PC differentiation. (B) Proportions of B220⁺, CD138⁺, CD44⁺, CD93⁺, TAC1⁺, IgD⁺, MHCII⁺ cells on day 7 of *in vitro* PC differentiation. (C) Expression levels of B220, CD138, CD44, CD93, TAC1, IgD, and MHCII in B220⁺CD138⁻ cells on day 7 of *in vitro* PC differentiation. Data are presented as mean \pm SD. Unpaired, 2-tailed Student's *t*-test (**p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001).

Efficient isotype switching and Ab secretion by *in vitro* differentiated PCs

During PC differentiation, B cells undergo class switch recombination to produce Abs with diverse Ig heavy chain constant regions associated with distinct functions (30). We next performed intracellular immunoglobulin analysis of B cells at day 7 using flow cytometry. Compared to CD138⁻ B cells, significant proportions of B220⁺CD138⁺ B cells expressed IgG1, suggesting successful immunoglobulin isotype switching in PCs generated using our *in vitro* culture conditions (Fig. 4A). We also measured Ab levels in the culture supernatants by ELISA and confirmed the secretion of IgM, IgG1, and IgG3, but not IgA (Fig. 4B), demonstrating successful isotype switching and efficient Ab secretion by the *in vitro* generated PCs.

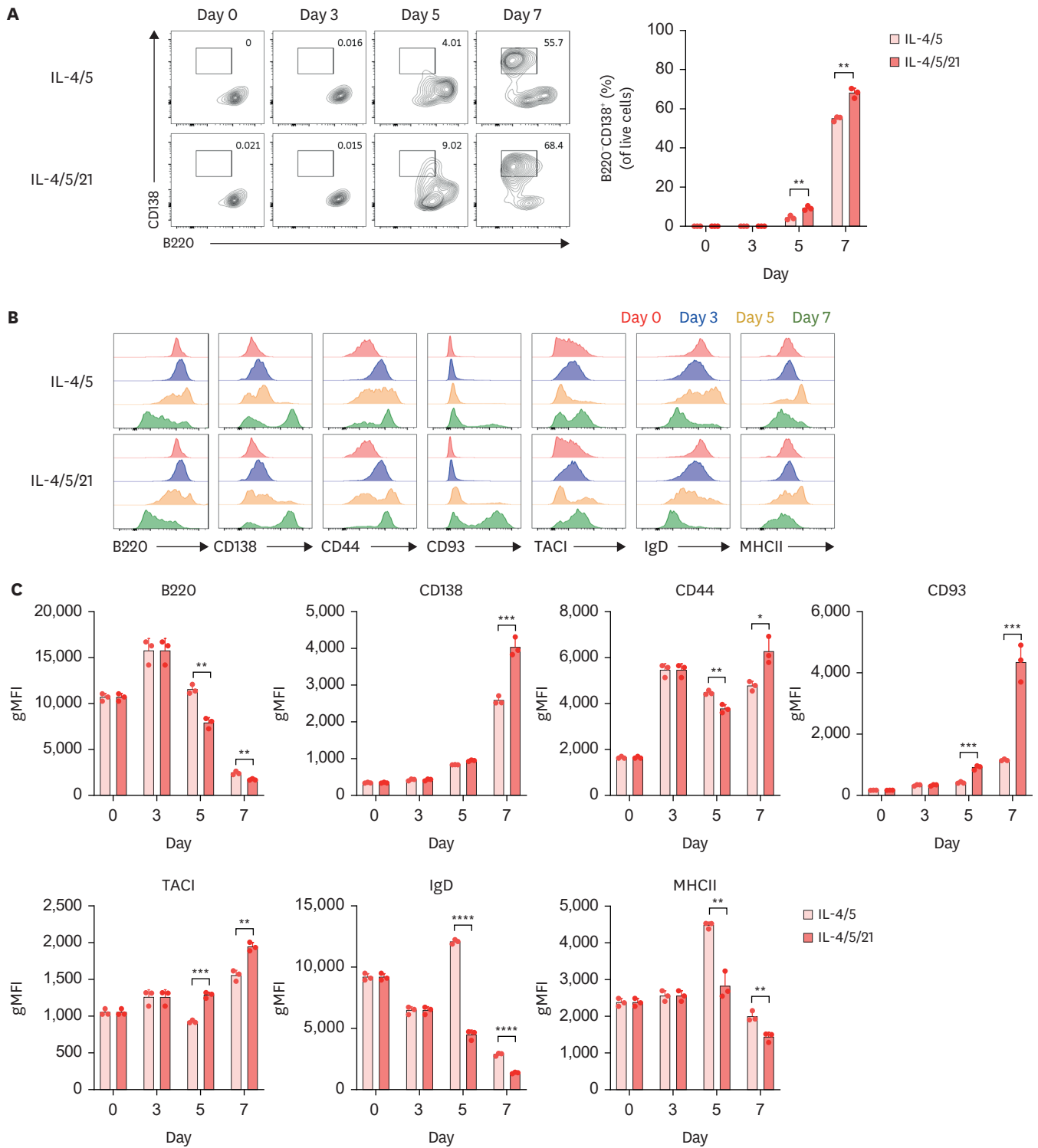


Figure 3. Time-course analysis of *in vitro* plasma cell differentiation by α -IgM/ α -CD40 and cytokine stimulation. (A) Proportions of B220⁺CD138⁺ cells on the indicated days of *in vitro* PC differentiation. (B) Expression levels of B220, CD138, CD44, CD93, TACI, IgD, and MHCII on the indicated days of *in vitro* PC differentiation. (C) gMFI values for B220, CD138, CD44, CD93, TACI, IgD, and MHCII on the indicated days of *in vitro* PC differentiation. Data are presented as mean \pm SD. Unpaired, 2-tailed Student's *t*-test (* p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001).

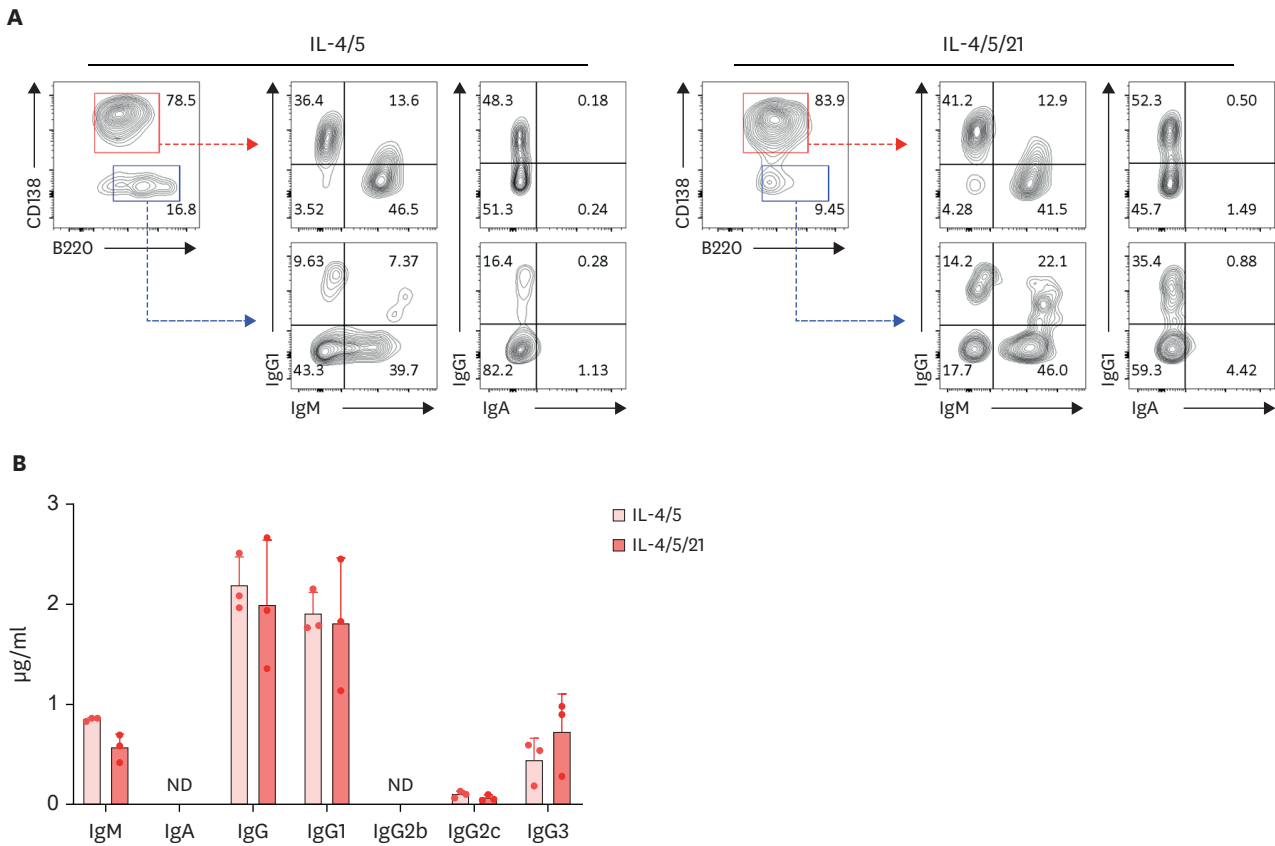


Figure 4. *In vitro* differentiated B cells undergo class switch recombination and secrete isotype-switched antibodies. (A) Intracellular immunoglobulin expression in B220⁺CD138⁺ and B220⁻CD138⁻ cell on day 7 of *in vitro* PC differentiation. (B) Levels of each antibody isotype in the culture supernatant on day 7 of *in vitro* PC differentiation. Data are presented as mean ± SD. ND, not detected.

Expression kinetics of PC lineage-determining factors during *in vitro* PC differentiation

The PC differentiation process is tightly regulated and finely tuned through complex gene expression dynamics. Over the years, critical regulators of PC differentiation have been identified, governing B cell fate at specific time points (5). Therefore, we next examined the gene expression kinetics of key regulatory molecules associated with B cell differentiation. As expected, naïve B cells exhibited high expression of *Pax5* and *Bach2*, which are markers of naïve follicular B cells (Fig. 5A and B). Their expression levels rapidly decreased following stimulation with α -IgM/CD40 and remained low until day 7. The expression of *Aicda*, a critical gene for the initiation of class switch recombination, was transiently increased after cytokine stimulation, peaking at days 4 and 5, returning to basal levels by day 7 (Fig. 5C). Additionally, cytokine stimulation induced upregulation of PC fate-determining transcription factors, such as *Irf4*, *Prdm1*, and *Xbp1* (Fig. 5D-F). Their expression increased as early as day 4, peaked at day 6, and slightly decreased by day 7. Expression of *Jchain*, required for multimeric secretory Ab production, was induced starting at day 5 and continuously increased until day 7 (Fig. 5G). The induction of *Irf4*, *Prdm1*, *Xbp1*, and *Jchain* began earlier, and the magnitude of their expression was higher in IL-4/5/21-stimulated cells compared to IL-4/5-stimulated cells. These findings align with the results showing higher PC differentiation efficiency in the IL-4/5/21-stimulation condition in Fig. 1C. Collectively, PCs generated through our 2-step *in vitro* PC differentiation method, incorporating BCR activation and cytokine stimulation, reliably recapitulate the

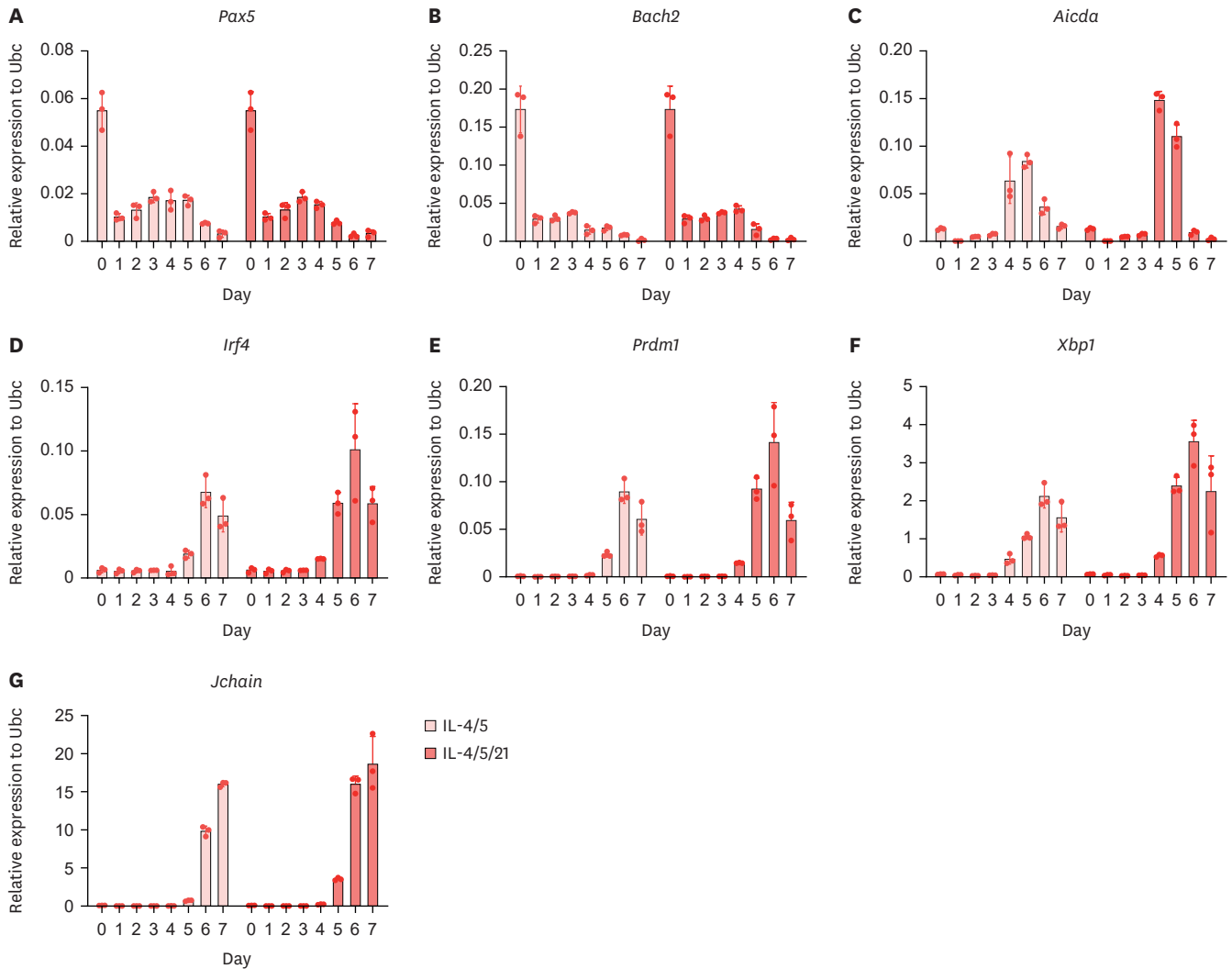


Figure 5. *In vitro* plasma cell differentiation by α -IgM/ α -CD40 and cytokine stimulation induces the expression of core genes for plasma cell differentiation and function. qRT-PCR analysis of *Pax5* (A), *Bach2* (B), *Aicda* (C), *Irf4* (D), *Prdm1* (E), *Xbp1* (F), and *Jchain* (G) on the indicated days of *in vitro* PC differentiation. Data are presented as mean \pm SD.

characteristics of natural PCs generated *in vivo*, as evidenced by various analyses of PC surface markers, transcription factors, intracellular immunoglobulins, and secreted Abs.

Efficient retroviral gene transduction of B cells during *in vitro* PC differentiation

PCs are usually intractable to genetic engineering due to their terminally differentiated status and low proliferation capacity. However, genetically modified PCs can be generated by retroviral gene transduction of activated B cells during *in vitro* PC differentiation process (31,32). To test the feasibility of transgene expression in PCs generated using our PC differentiation method, we infected activated B cells with ecotropic retroviruses encoding GFP on day 1 post- α -IgM/CD40 stimulation (Fig. 6A). When GFP-positive cells were analyzed by flow cytometry on day 7, approximately 50% of total live cells expressed GFP (Fig. 6B). Moreover, both GFP-expressing and GFP-negative cells showed comparable frequencies of B220⁺CD138⁺ population (Fig. 6C), indicating that viral transduction had minimal impact on PC differentiation efficiency. Therefore, our 2-stage *in vitro* PC differentiation method is highly compatible with retrovirus-mediated genetic engineering.

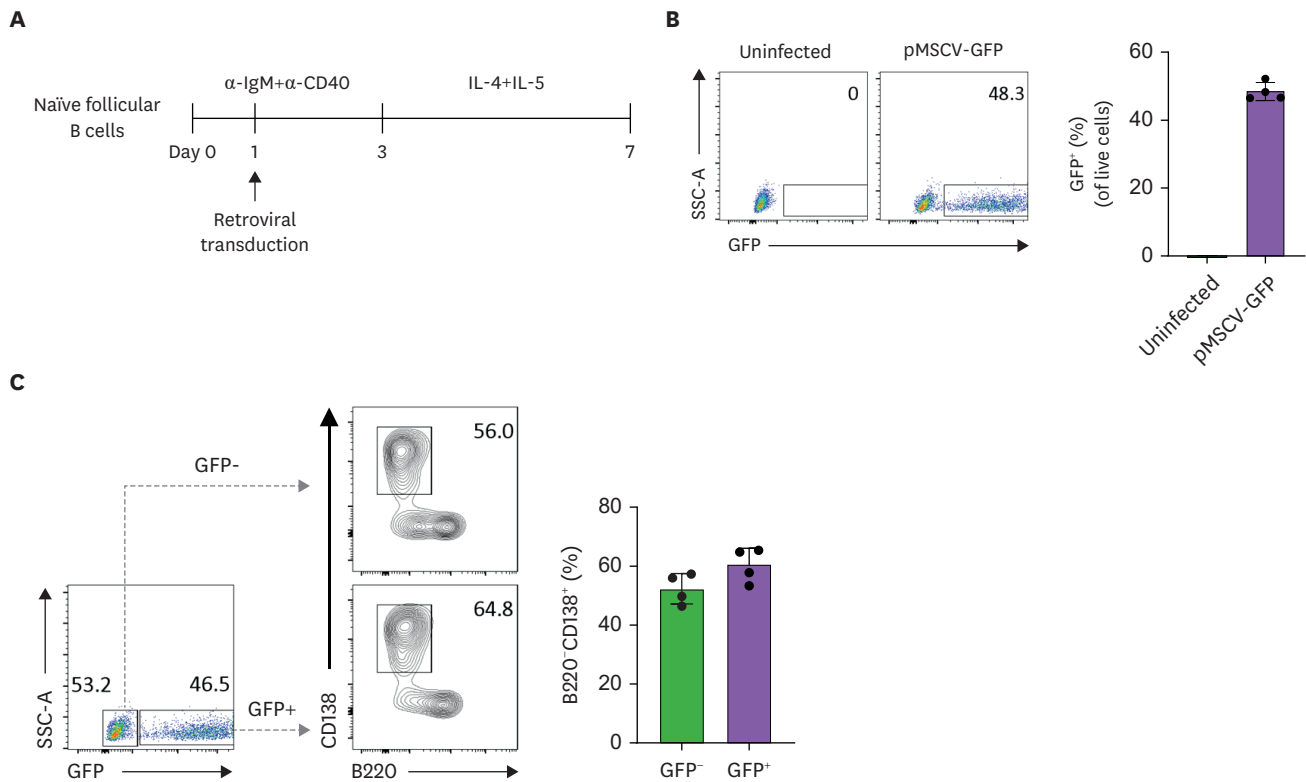


Figure 6. Efficient transgene expression in plasma cells by retroviral transduction during *in vitro* plasma cell differentiation. (A) Experimental scheme for retroviral transduction during *in vitro* PC differentiation. (B) Proportions of GFP-positive B cells on day 7 of *in vitro* PC differentiation. (C) Proportions of B220⁺ CD138⁺ cells among GFP-negative and GFP-positive cells on day 7 of *in vitro* PC differentiation.

DISCUSSION

In this study, we developed a novel culture method to efficiently produce a large quantity of PCs from naïve follicular B cells. By applying a combination of BCR and CD40 stimulation, we effectively mimicked the Ag-mediated BCR activation and T cell-mediated CD40 activation that occur during T cell-dependent PC differentiation *in vivo*. This dual stimulation method, followed by cytokine-driven differentiation, resulted in robust proliferation and efficient differentiation of naïve B cells into PCs. These PCs expressed key surface markers and transcription factors associated with PC identity and were capable of secreting isotype-switched Abs. Furthermore, this method is compatible with retroviral transduction, allowing for the stable expression of recombinant proteins, making it a valuable tool for studying PC differentiation and related mechanisms.

Unlike most existing *in vitro* PC differentiation protocols, our method includes BCR stimulation, which is the first critical event in natural PC differentiation. However, BCR signaling alone is known to induce apoptosis in mature naïve B cells unless accompanied by co-stimulatory signals such as CD40, IL-4, or TLR agonists (33-35). Additionally, BCR signaling by itself does not promote PC differentiation; instead, it can inhibit class switch recombination by delaying the expression of *Aicda* and antagonize PC differentiation (36,37). By combining BCR and CD40 activation, naïve B cells were rescued from BCR activation-induced apoptosis, actively proliferated, and underwent efficient PC differentiation following cytokine stimulation (Fig. 1B).

For BCR activation, we used anti-IgM F(ab')₂ Ab from Jackson ImmunoResearch Laboratories (Cat. No. 115-006-075). Interestingly, we were unable to induce PC differentiation using the same concentration of anti-IgM F(ab')₂ Ab obtained from SouthernBiotech (Cat. No. 1022-01), even though Abs from both vendors effectively induced B cell activation and proliferation (data not shown). Since the strength of BCR signaling significantly influences PC differentiation (38-40), variations in BCR signaling strength caused by different anti-IgM Abs could have affected the outcome of PC differentiation. Although we did not directly compare the BCR signaling strength elicited by each reagent, we speculate that there is an optimal range of BCR signaling strength for efficient *in vitro* PC differentiation. Indeed, previous studies found that the maximum IRF4 expression and PC differentiation are dependent on the concentration of Ags used to activate B cells (38,40). Therefore, careful titration of anti-IgM F(ab')₂ may result in a more refined *in vitro* PC differentiation method with an even higher yield.

Notably, B cells activated with α -CD40 alone, in the absence of α -IgM, were still able to differentiate into B220⁻CD138⁺ cells following IL-4/5 or IL-4/5/21 stimulation (Fig. 1C). However, the final yield of B220⁻CD138⁺ PCs was significantly lower under these conditions compared to those with α -IgM/CD40 stimulation, primarily due to the lack of B cell proliferation during the 3 day α -CD40 stimulation period (Fig. 1B). Additionally, we suspect that not only the quantity but also quality of B220⁻CD138⁺ PCs may differ between α -IgM/CD40 and α -CD40 stimulation groups. A previous study demonstrated that PCs generated using a culture condition incorporating BCR stimulation exhibited a unique gene expression profile and differential *Prdm1* promoter usage compared to PCs differentiated without BCR activation (41). Moreover, transcriptomic analyses revealed that the former group, but not the ASCs generated by *in vitro* culture with CD40L, IL-4, and IL-5, closely resembled *ex vivo* PCs from the bone marrow and spleen (41,42). Nevertheless, the precise molecular mechanisms by which the BCR signaling pathway regulates the PC differentiation process remains to be elucidated.

IL-4 and IL-5 are widely recognized for their roles in promoting B cell growth and differentiation (26-28). We found that neither IL-4 nor IL-5 alone could induce efficient PC differentiation of activated B cells; however, the combined stimulation with IL-4 and IL-5 led to robust differentiation of PCs (Fig. 1C). A previous study reported that combined treatment with IL-4 and IL-5 effectively induced IgG1 class switching in B cells activated with anti-IgD-conjugated dextran, while either IL-4 or IL-5 alone was insufficient to trigger class switch recombination (43). Additionally, transcriptomic analysis revealed that IL-4 and IL-5 drive distinct gene expression profiles, suggesting differential requirements for these cytokines in PC differentiation (44). Specifically, only IL-5 was able to induce *Prdm1* and *Jchain* expression, whereas both IL-4 and IL-5 promoted the expression of *Aicda* and *Xbp1* (44). It is unclear why IL-5 alone is insufficient to induce *in vitro* PC differentiation of activated B cells, even though it can apparently induce key regulators of PC differentiation and isotype switching. The role of IL-5 in B cell-mediated Ab responses *in vivo* also remains controversial. IL-5-transgenic mice displayed elevated serum IgM levels and spontaneous production of autoantibodies (45). On the other hand, IL-5-deficient mice exhibited no significant defects in systemic Ab production, either under steady-state condition or after immunization (46). Therefore, the exact contributions of IL-4 and IL-5 in *in vitro* PC differentiation need further elucidation.

Stimulation with IL-4/5 of α -IgM/CD40-activated B cells immediately initiated the PC differentiation program, beginning with the transient expression of *Aicda*, followed by gradual expression of *Irf4*, *Prdm1*, *Xbp1*, and *Jchain* (Fig. 5D-G). The addition of IL-21 to the IL-4/5 combination resulted in faster and more potent expression of these genes and a higher

proportion of B220⁺CD138⁺ PCs among live B cells (**Fig. 1C**), which is consistent with the well-known role of IL-21 in promoting PC differentiation (47,48). However, including IL-21 in our culture conditions led to a drastic decrease in cell viability by day 5 (**Fig. 1B**). Consequently, the final number of PCs differentiated by IL-4/5/21 stimulation was less than half of that generated by IL-4/5 stimulation. IL-21 has been shown to induce apoptosis in activated B cells in the absence of concurrent CD40L stimulation (29,49). On the other hand, the expression of PC-associated surface markers and core genes was similar between PCs differentiated with IL-4/5 and IL-4/5/21. A previous study also showed that *in vitro* differentiated PCs without IL-21 still closely resembled PCs generated *in vivo* (41). Therefore, the addition of IL-21 during our *in vitro* PC differentiation appears to be unnecessary.

Unlike IL-4 and IL-5, we were unable to induce PC differentiation using IL-2 and IL-10, which are often used for PC differentiation of human B cells (50-52). The differential requirements for PC differentiation between human and mouse B cells are not well-defined, and further investigation is needed to determine if other cytokines can potentially induce PC differentiation of murine B cells. Our method, described here, can be easily adapted for screening of cytokines and other environmental factors to enhance PC differentiation, as well as to facilitate Ab class switching to a specific isotype of interest.

Recently, a couple of studies reported *in vitro* PC differentiation using transgenic B1-8 B cells expressing the NP-specific BCR heavy chain (38,40). By simultaneously stimulating B1-8 B cells with IL-2, IL-4, IL-5, α -CD40, and NP, the authors were able to generate up to 50% of CD138-positive cells (40). In another study, wild-type naïve B cells were activated with a combination of α -IgM/IgG, IL-4, IL-5, and CD40L for just 1 day, and then cultured in media containing IL-6 and APRIL, resulting in approximately 80% of B220⁺CD138⁺ cells by day 13 (41). Although the proportion of PCs in the final culture was relatively high, the absolute number of PCs on day 13 was similar to the initial numbers of naïve B cells seeded at the start of the culture, likely due to limited cell proliferation under this condition. Moreover, these PCs secreted much less IgG than IgM, possibly because CD43-negative selection of splenocytes was used for naïve B cell preparation, resulting in a mixed population of follicular, marginal zone, and transitional B cells (41). Compared to these previous methods, our new method does not require the use of transgenic mice and produces a high yield of isotype-switched PCs within a week.

B cells have generally been thought to be resistant to genetic manipulation via commonly used viral transduction methods. However, by using retrovirus with ecotropic envelope proteins, we were able to achieve stable and high expression of a target gene in PCs. This viral transduction method can also be applied for shRNA-mediated gene knock-down or CRISPR/Cas9-mediated manipulation of gene expression. Combined with our simple *in vitro* PC differentiation method, this approach provides a powerful system for identifying crucial regulators of the BCR signaling-dependent PC differentiation.

In summary, our new 2-step *in vitro* PC differentiation method offers a simple yet effective system to quickly produce large quantities of PCs for various applications, including biochemical analysis and elucidation of the molecular mechanisms underlying PC differentiation.

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