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Short communication

Isolation and characterization of naïve follicular dendritic cells

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Abstract

Follicular dendritic cells (FDC) are specialized antigen-presenting cells to cognate B cells in the follicle of the lymphoid tissues. FDC also support survival and proliferation of the B cells, leading to the germinal center formation. FDC therefore play a central role in humoral immune responses. However, molecular and functional characteristics of FDC are largely unknown, because it is difficult to isolate and analyze FDC due to a very small number of FDC in the lymphoid tissues and the fragility by mechanical and chemical stresses in vitro. In this report, we established a novel method for FDC isolation from the spleen of naïve mice by flow cytometry and analyzed the phenotypical and functional characteristics. The isolated FDC, which accounted for ~ 0.2% of the spleen cells of naïve mice, were CD45⁻, FDC-M2⁺, and ICAM-1⁺, and supported the survival and LPS-induced proliferation of B cells. We also showed that a neutralizing antibody against B cell activating factor TNF family (BAFF) suppressed FDC-dependent B cell proliferation in the presence of LPS, but not survival, demonstrating the evidence that FDC-derived BAFF is involved in B cell proliferation.

Keywords;

FDCs (Follicular dendritic cells), BAFF (B cell activating factor TNF family)

1. Introduction

Most B cells are located inside the follicles of the lymphoid tissues, which segregate the direct access of blood-borne antigens to B cells. Previous reports demonstrated that the distribution of an immune complex inside the lymphoid follicles was reticular fashion, and this observation led to the identification of specialized antigen-trapping cells, named follicular dendritic cells (FDC) (Chen et al., 1978a; Chen et al., 1978b; Nossal et al., 1968). FDC have the ability to trap and retain surface bound immune complex by the complement receptors 1/2 (CD21/35) and Fc γ RIIb. FDC also support survival and proliferation of B cells, leading to the germinal center (GC) formation. Thus, FDC are pivotal for GC-related humoral immune responses, including immunoglobulin class-switching, somatic hypermutation of B cell receptor gene for affinity maturation, and generation of memory B cells (Imal and Yamakawa, 1996; Qin et al., 2000; Tew et al., 1997; Yoshida et al., 1993). However, molecular and functional characteristics of FDC are largely unknown, because it is difficult to isolate and analyze FDC due to a very small number of FDC in the lymphoid tissues and the fragility by mechanical and chemical stresses in vitro.

Previous studies demonstrated that FDC were isolated from the lymph nodes after reducing lymphocytes by whole body irradiation of mice to increase the frequency of FDC. Moreover, mice were primed with an antigen before FDC isolation to generate GC (Kranich et al., 2008; Sukumar et al., 2006; Suzuki et al., 2010). However, it is essentially required to isolate FDC from naïve mice for understanding the physiological function of FDC in humoral immune responses.

In this report, we established a novel method for FDC isolation from the spleen of naïve mice by flow cytometry. By using the isolated naïve FDC, we showed the direct evidence that FDC-derived BAFF is involved in B cell proliferation *in vitro*.

2. Materials and Methods

2.1. Mice

C57BL/6J mice were purchased from Clea Japan Inc. (Tokyo, Japan) and maintained under the specific pathogen-free (SPF) condition. All experiments were performed according to the guidelines of the Animal Ethics Committee of the University of Tsukuba Animal Research Center.

2.2. Antibodies

Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (mAb) against the cell surface molecules, including CD40, CD54, CD16/32, CD80, CD86, CD106, CD11b, CD11c, NK1.1, CD45R/B220, TCR β , CD21/35, and allophycocyanin (APC)-conjugated streptavidin were purchased from Pharmingen (San Diego, CA, USA). Biotin-conjugated anti-FDC-M2 mAb was purchased from ImmunoKontakt (Abingdon, UK). Phycoerythrin (PE)- and biotin-conjugated anti-CD45.2 mAb was purchased from Biolegend (San Diego, CA, USA). Purified anti-Mfge8 Ab (2422) was purchased from MBL (Nagoya, Japan). Purified anti-LT β R Ab was purchased from

eBioscience (San Diego, CA, USA). Alexa546-conjugated goat anti-rat IgG and Alexa546-conjugated streptavidin were purchased from Invitrogen Life technologies (Carlsbad, CA, USA). Goat anti-mouse BAFF mAb was purchased from R&D systems (Minneapolis, MN, USA).

2.3. Isolation of FDC

The spleen from naïve mice was fragmented into small pieces by a scalpel and then treated with the enzyme cocktail of 33.3 mg/ml collagenase D (Roche, Indianapolis, IN, USA) and 2500 U/ml DNase I (Sigma, St. Louis, MO, USA) in complete DMEM medium containing 2% fetal bovine serum (FBS). After incubation for 1 h at 37 °C in a humidified incubator, cells were dissociated by pipetting and then stained with biotin-conjugated anti-FDC-M2 mAb, followed by APC-conjugated streptavidin, PE-conjugated anti-CD45.2 mAb and FITC-conjugated mAb, and analyzed by FACS Calibur (Becton Dickinson, San Diego, CA, USA). To purify FDC, CD45⁻ cells were enriched by staining biotin-conjugated anti-CD45.2 mAb, followed by streptavidin particles plus-DM (BD Pharmingen, San Diego, CA, USA), according to the

manufacturer's instruction. Cells were then again stained with PE-conjugated anti-CD45.2 mAb and FITC-conjugated anti-ICAM-1 mAb, and FDC, as defined by CD45.2⁻, ICAM-1⁺ cells, were purified by sorting with FACS Aria (Becton Dickinson).

2.4. Polymerase chain reaction

PCR analysis was carried out by using the PCR primers specific for *Mfge8* (5'-ATATGGGTTTCATGGGCTTG-3' and 5'-GAGGCTGTAAGCCACCTTGA-3'), *Cd54* (5'-GTCATGCTCAGGTATCCATCCA-3' and 5'-CACAGTTCTCAAAGCACAGCG-3') and *Hprt* (5'-GCTGGTGAAAAGGACCTCT-3' and 5'-CACAGGACTAGAACACCTGC-3').

2.5. Immunostaining of FDC

Purified CD45.2⁻, FDC-M2⁺, ICAM-1⁺ cells (1 x 10⁵) were cultured in the collagen type I-coated 8-well chamber slide (Becton Dickinson) in the presence of TNF α (5 ng/ml) and anti-LT β R (1 μ g/ml) agonist Ab in complete DMEM medium containing 10 % FBS for 7 days. Cells were fixed with acetone and stained with anti-Mfge8 mAb, followed

by Alexa-546-conjugated goat anti-hamster IgG, or biotin-conjugated anti-FDC-M2 mAb or biotin-conjugated anti-Fc α / μ R, followed by Alexa 546-conjugated streptavidin, along with FITC-conjugated anti-CD54 mAb and Alexa 647-conjugated anti-CD21/35 mAb. Samples were analyzed under a fluorescence microscope.

2.6. Survival and proliferation assays

Purified CD45.2⁻, FDC-M2⁺, ICAM-1⁺ cells ($3\sim 10 \times 10^4$) or CD45.2⁻, FDC-M2⁻, ICAM-1⁻ cells ($3\sim 10 \times 10^4$) were cultured in the collagen type I-coated 96-well plate in the presence of TNF α (5 ng/ml) and anti-LT β R agonistic mAb (1 μ g/ml) in complete DMEM medium containing 10 % FBS for 7 days. Naïve B cells (2×10^5) purified from the spleen by using anti-B220 particles-DM (BD Pharmingen), according to the manufacturer's instruction, were labeled with 50 nM CFSE. For survival assay, CFSE-labeled B cells were added into each culture of FDC and co-cultured for three days. Cells were harvested and the frequencies of CFSE⁺, PI⁻ cell population were determined by FACSCalibur. For proliferation assay, CFSE-labeled B cells (2×10^5) were co-cultured with FDC for three days in the absence or presence of

lipopolysaccharides (LPS) (Sigma, St. Louis, MO, USA). Three days later, cells were harvested and stained with APC-conjugated anti-B220 mAb. The proliferation of B220⁺ cells was analyzed by CFSE dilution assay. For BAFF neutralization, 10 µg/ml anti-BAFF mAb or a control goat IgG was added into the culture.

2.8. Statistics

Statistical analyses were performed by using Student's unpaired *t*-test. The Smirnov-Grubbs test was used to exclude outliers.

3. Results and Discussion

3.1. Isolation of naïve FDC

In previous reports, FDCs were isolated from the lymph nodes of antigen-primed mice by using FDC-specific marker, FDC-M1, also known to be as milk fat globular protein EGF-8 (MFG-E8) (Kranich et al., 2008). However, FDC-M1 expression on FDCs was faint in naïve mice and upregulated during germinal center formation (Camacho et al., 1998; Martinez-Pomares et al., 1996), suggesting that FDC-M1 may not be a good marker for naïve FDC. Instead, we examined whether FDC-M2 and/or ICAM-1, also reported to be expressed on FDC, could be markers for naïve FDC. Since FDC are not derived from the hematopoietic stem cell (Munoz-Fernandez et al., 2006), we examined FDC-M2⁺ and/or ICAM-1⁺ cells in CD45⁻ non-hematopoietic cell fraction. Spleen cells from naïve mice were treated with collagenase and the DNase enzyme cocktail, and simultaneously stained with mAbs against FDC-M2, ICAM-1 and CD45.2, demonstrating a very small population (0.2% (mean) \pm 0.06% (SD) in total spleen cells, n=6) of CD45⁻, FDC-M2⁺, ICAM-1⁺ cells (**Fig. 1A**). This cell population also expressed VCAM-1 (CD106), CD40, CD80 and CD16/32 moderately and lymphotoxin β receptor

(LT β R) and FDC-M1 weakly, but not the lineage markers B220, TCR β , NK1.1, CD11b and CD11c (**Fig. 1B**), consistent with the previous findings demonstrated by immunohistochemical analyses (Allen and Cyster, 2008; El Shikh et al., 2010).

3.2. Genetic and morphological analyses of CD45⁻, FDC-M2⁺, ICAM-1⁺ cells

We obtained $1.0\text{-}2.0 \times 10^5$ CD45.2⁻, FDC-M2⁺, ICAM-1⁺ cells and CD45.2⁻, FDC-M2⁻, ICAM-1⁻ cells with >90 % purity from the spleen of a mouse by flow cytometry (**Fig. 1C**). RT-PCR analysis showed the expression of *Mfge8* and *Cd54* in CD45.2⁻, FDC-M2⁺, ICAM-1⁺ cells, but not in CD45.2⁻, FDC-M2⁻, ICAM-1⁻ cells (**Fig. 1D**). CD45.2⁻, FDC-M2⁺, ICAM-1⁺ cells could be maintained on collagen-coated cell-culture slide more than one week in the presence of TNF α and LT β R agonistic antibody, essential factors for maturation and maintenance of FDC (El Shikh et al., 2006; Nishikawa et al., 2006; Suzuki et al., 2010). The cultured, CD45.2⁻, FDC-M2⁺, ICAM-1⁺ cells showed spindle shape with extended thin dendrites, which were entangled with each other as observed in FDC network (**Fig. 1E**), and strongly expressed FDC-M1, FDC-M2, CD21/35, and Fc α / μ R by immunohistochemical analyses (**Fig. 1E**).

3.3. Functional analyses of CD45⁻, FDC-M2⁺, ICAM-1⁺ cells

Naïve B cells were labeled with CFSE and cultured alone or co-cultured with either CD45.2⁻, FDC-M2⁺, ICAM-1⁺ cells or CD45.2⁻, FDC-M2⁻, ICAM-1⁻ cells for 3 days.

The cultured cells were stained with anti-B220 mAb and propidium iodide (PI), and the frequencies of live B cells, as defined by PI⁻, CFSE⁺, B220⁺ cells, were determined by flow cytometry. The frequency of live B cells was significantly higher when co-cultured with CD45.2⁻, FDC-M2⁺, ICAM-1⁺ cells than with CD45.2⁻, FDC-M2⁻, ICAM-1⁻ cells (**Fig. 2A**), suggesting that CD45.2⁻, FDC-M2⁺, ICAM-1⁺ cells supported B cell survival.

Next, CFSE-labeled naïve B cells were stimulated with LPS alone or in the presence of either CD45.2⁻, FDC-M2⁺, ICAM-1⁺ cells or CD45.2⁻, FDC-M2⁻, ICAM-1⁻ cells for 3 days and the proliferation of B cells were determined by CFSE dilution assay. B cell proliferation was significantly increased when co-cultured with CD45.2⁻, FDC-M2⁺, ICAM-1⁺ cells, compared to the proliferation of B cells co-cultured with CD45.2⁻, FDC-M2⁻, ICAM-1⁻ cells (**Fig. 2B**), indicating that CD45.2⁻, FDC-M2⁺, ICAM-1⁺ cells supported B cell survival and LPS-induced proliferation of B cells. Taken together, the

phenotypical, morphological and functional characteristics of CD45.2⁻, FDC-M2⁺, ICAM-1⁺ cells were consistent with those of FDC.

3.4. BAFF derived from CD45⁻, FDC-M2⁺, ICAM-1⁺ cells support B cells proliferation.

Although BAFF secreted by FDC is thought to be involved in B cell survival and proliferation in the lymphoid tissues (Gorelik et al., 2003; Lesley et al., 2004; Nishikawa et al., 2006; Zhang et al., 2005), no report has demonstrated the direct evidence for this notion in vitro. To address this issue, we added a neutralizing mAb against BAFF into the co-culture of CFSE-labeled B cells with CD45⁻, FDC-M2⁺, ICAM-1⁺ cells. We observed that anti-BAFF mAb significantly suppressed the LPS-induced proliferation, but not survival, of B cells co-cultured with CD45⁻, FDC-M2⁺, ICAM-1⁺ FDC (**Fig. 2C, D**). In contrast, anti-BAFF mAb did not affect the LPS-induced proliferation of B cells that were cultured alone or co-cultured with CD45⁻, FDC-M2⁻, ICAM-1⁻ cells, indicating that BAFF was produced from CD45⁻, FDC-M2⁺, ICAM-1⁺ FDC and stimulated LPS-induced B cell proliferation.

In conclusion, we established a novel method for isolation of naïve FDC from the spleen by using the cell surface markers CD45⁻, FDC-M2⁺, ICAM-1⁺ by flow cytometry. We demonstrated that the isolated FDC supported B cell survival and LPS-induced proliferation in vitro, and provided for the first time the direct evidence that BAFF produced by FDC supported LPS-induced B cell proliferation, but not survival, in vitro. It was likely that B cell survival was dependent on anti-apoptotic signals through cell-cell interaction with FDC (Koopman et al., 1994; Koopman et al., 1991), but independent of soluble proteins from FDC. The isolated naïve FDC should be useful for molecular and cellular analyses of humoral immune responses in vitro.

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Figure Legends**Fig. 1. Isolation and phenotypical and morphological characterization of naïve FDC**

(A) Spleen cells from naïve mice were treated with the enzyme cocktail, as described in Materials and Methods, and stained with biotinylated-anti-FDC-M2 mAb followed by APC-conjugated streptavidin, PE-conjugated anti-CD45.2 mAb, FITC-conjugated anti-ICAM-1 mAb, and PI. The percentages of the PI⁻, FDC-M2⁺, ICAM-1⁺ cell population within the total PI⁻ cell fraction were shown. (B) Naïve spleen cells were also stained with biotinylated-anti-FDC-M2 mAb followed by APC-conjugated streptavidin, PE-conjugated anti-CD45.2 mAb, FITC-conjugated mAb against each molecule indicated, and PI. The expressions of each molecule on the CD45.2⁻, FDC-M2⁺, ICAM-1⁺ gate are shown. (C-E) CD45.2⁻, FDC-M2⁺, ICAM-1⁺ cells or CD45.2⁻, FDC-M2⁻, ICAM-1⁻ cells were sorted by flow cytometry (C) and subjected to RT-PCR (D) and immunohistochemical analyses (E).

Fig. 2. Functional analyses of CD45⁻, FDC-M2⁺, ICAM-1⁺ cells

CFSE-labeled B cells were or were not co-cultured with either CD45⁻, FDC-M2⁺, ICAM-1⁺ cells or CD45⁻, FDC-M2⁻, ICAM-1⁻ cells in the absence (A-D) or presence (B, D) of LPS for 3 days. Anti-BAFF mAb was also added into the culture (C, D). Cells were harvested and stained with anti-B220 mAb, and B220⁺ cells were examined for survival and proliferation by flow cytometry. (A, C) Cells on the B220⁺ cell gate (upper panel in A) and the frequencies of PI⁻ live B cells (lower panel in A, C) are shown (n=3). (B, D) B cell proliferation was analyzed by CFSE-dilution assay. The upper panel in A shows representative data of flow cytometry when B cells were cultured in the presence of LPS (500 ng/ml). The lower panel in B and D shows the frequencies of B cell that underwent cell division more than once. Data are representative of at least three independent experiments. Error bars indicate SD.

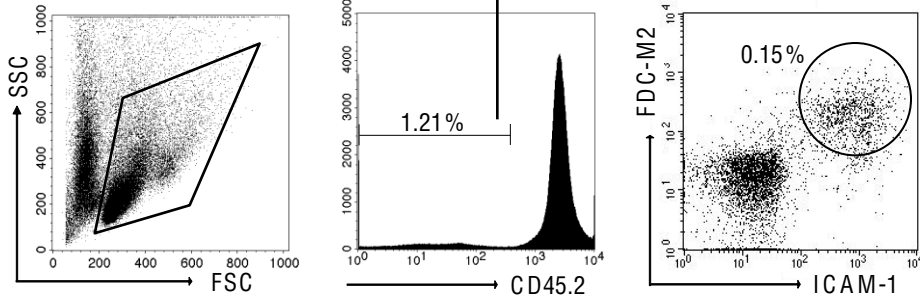
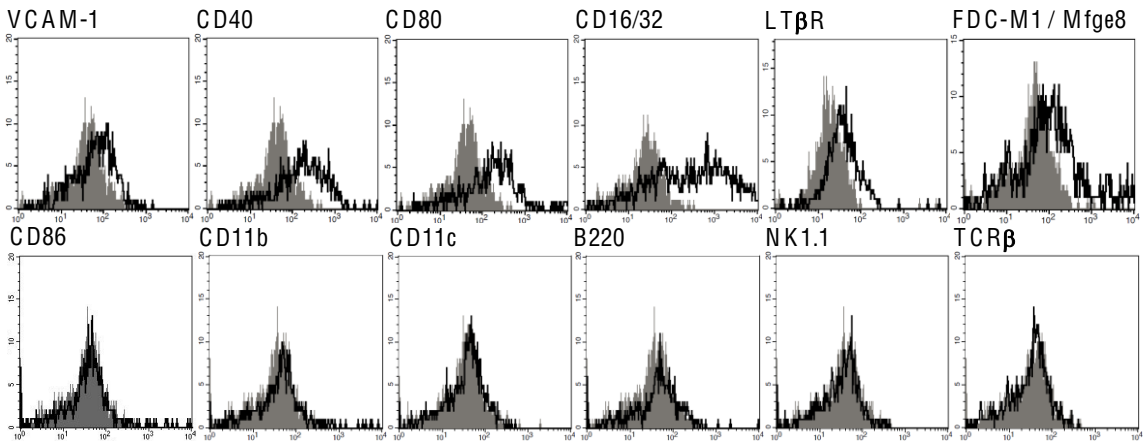
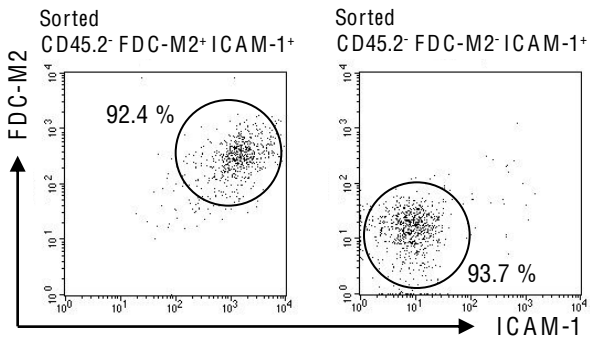
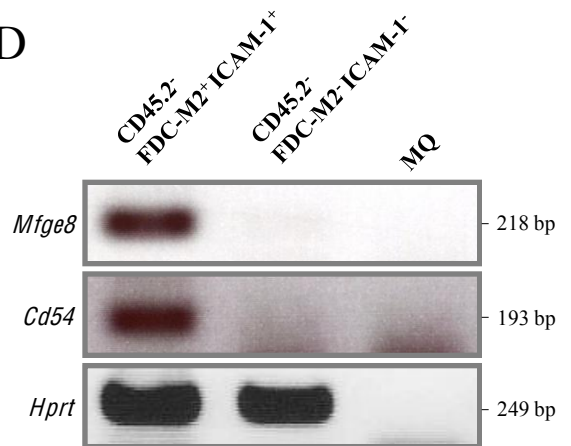
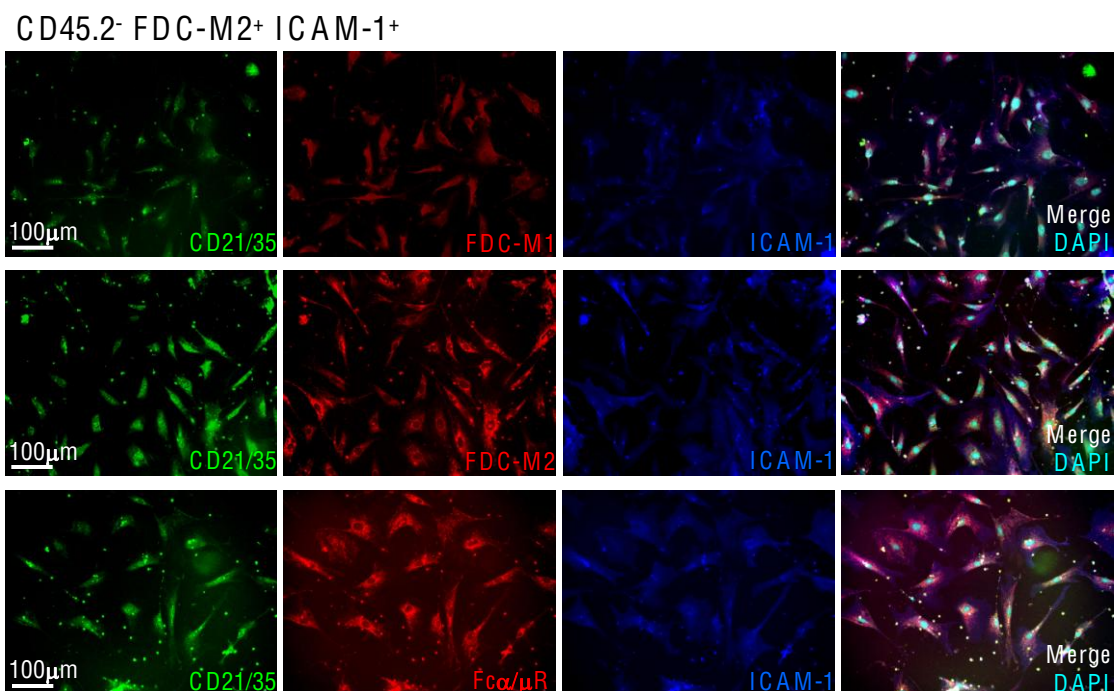
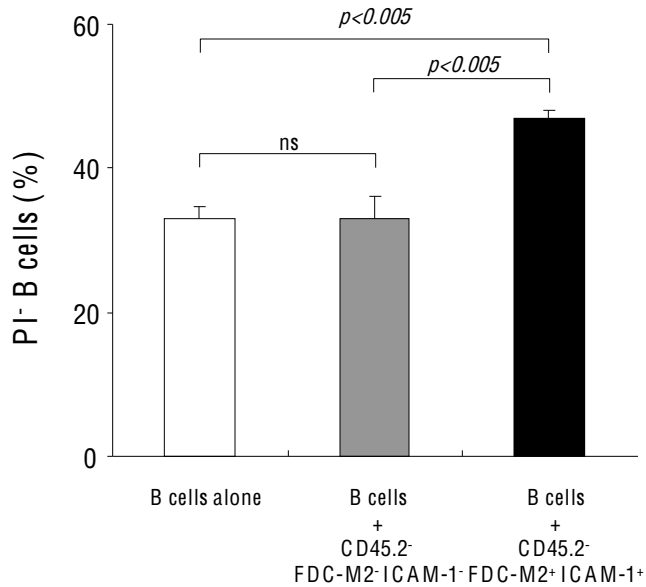
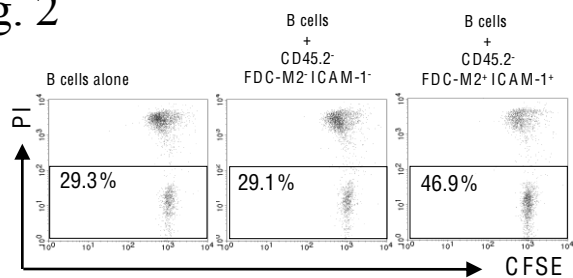
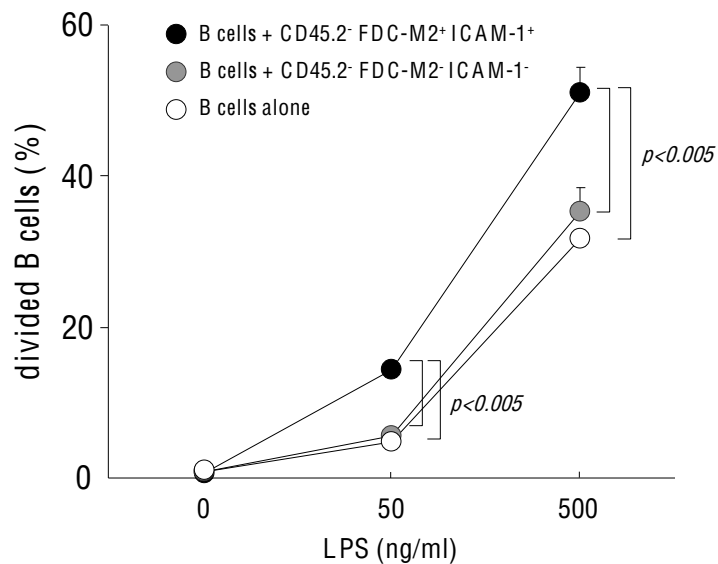
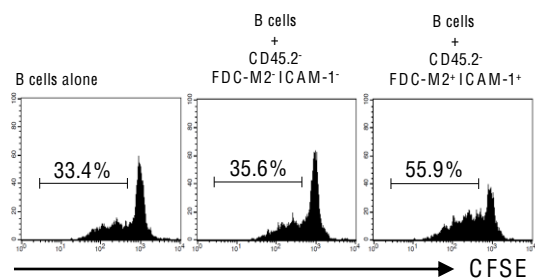
Figure 1**A****B****C****D****E**

Fig. 2

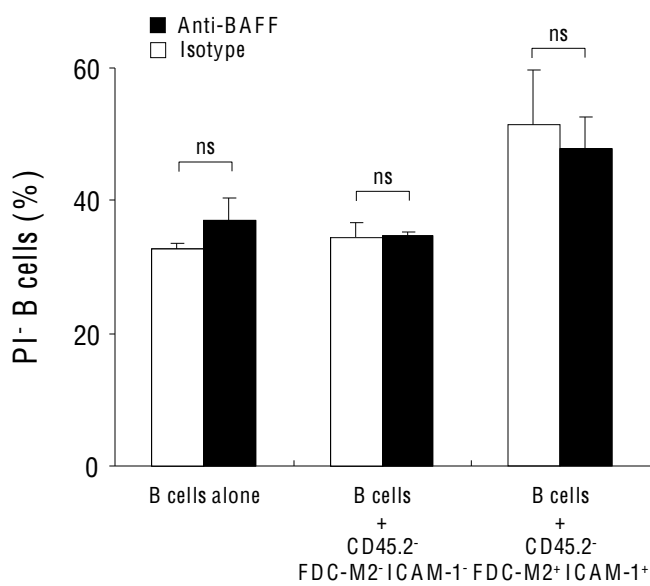
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